Tumor cell-extracellular matrix interactions : studies with antibodies to basement membrane components

TUMOR CELL-EXTRACELLULAR MATRIX INTERACTIONS: STUDIES WITH ANTIBODIES TO BASEMENT MEMBRANE COMPONENTS

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, prof.dr. F.I.M. Bonke,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen op vrijdag, 24 juni 1988 om 16.00 uur

door

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geboren te Kerkrade in 1950

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CHAPTER 1

General Introduction *

1.1 INTRODUCTION

Basement membranes (BM) occur almost everywhere in the body. They are found where epithelial cells border mesenchymal stroma. They connect vascular endothelium with the surrounding matriceal and cellular components of the vessel wall. They surround individual fat cells, Schwann cells, smooth muscle cells and cross striated muscle fibers and separate mesothelial cells from the adjacent connective tissue (1). Several functions are ascribed to BM. In structures such as nerves and in smooth, skeletal and cardiac muscle they provide strong but flexible physical support. In parenchymatous organs they serve as a scaffold for the epithelial cells (2-4). Furthermore they modulate the differentiation of epithelial cells, which is of particular importance during embryonal development (5). In the kidney they function as a semipermeable filtration barrier (6, 7).
In recent years many investigators have studied BM in a variety of neoplasms. Most studies have concerned the structural alterations in the BM in relation to infiltrative growth. It has been shown that in most carcinomas BM are fragmented and penetrated by invasive neoplastic epithelial cells (8). This phenomenon is of interest for at least two reasons. Firstly, knowledge of the mechanisms which lead to BM disintegration during invasive growth might shed some new light on the aberrant behavior of invasive neoplastic cells. Secondly, absence or fragmentation of the BM may be a useful criterium to distinguish between benign or in situ malignant and invasive neoplasms. In this review we will briefly discuss the structure and composition of BM, and provide a comprehensive overview of the current knowledge of BM alterations in neoplasia.

1.2 BASEMENT MEMBRANE STRUCTURE

The nomenclature concerning the BM is somewhat inconsistent in the literature. For practical purposes in this thesis we will use the term BM as synonymous with basal lamina, which in most BM is a combination of a lamina rara (lucida) and a lamina densa. By light microscopy BM, which can be more or less selectively stained by the PAS method or silver impregnation techniques,(9, 10) appear as thin structures

* Based on:
Basement membranes in cancer
Bosman FT, Havenith MG, Cleutjens JPM
Ultrastructural Pathology 1985; 8: 291-304

separating the cells from the adjacent connective tissue. By electron microscopy the structure of BM varies somewhat in relation to the cell type with which it is associated. With conventional (uranium and lead) staining most BM show a zone of low electron density adjacent to the cell membrane (lamina lucida or lamina rara) and a zone of higher electron density adjacent to the connective tissue (lamina densa), both of 40-60 nm thickness (1). Some BM, as for example the lens capsule or Descemet's membrane, consist of a lamina densa only (11). Most other BM contain a lamina rara as well as a lamina densa whereas occasionally, e.g. in the glomerulus or alveolus, the BM is trilaminar due to the juxtaposition of epithelial and endothelial BM (12, 13).

1.3 BASEMENT MEMBRANE COMPOSITION

BM are relatively insoluble and therefore biochemical analysis of some of the components has been rather complicated. The availability of transplantable neoplasms which produce large quantities of BM matrix, such as the EHS sarcoma (14) and the parietal yolk sac tumor, (15, 16) has facilitated the study of the chemical structure of its components. The human placenta has served as a readily available source of BM of a more physiological nature (17, 18).

Many substances have been found in BM. A number of these, e.g., fibronectin, type V collagen and entactin, do not occur exclusively in BM (19-21) and are not, or not only as is the case for entactin, synthesized by epithelial and endothelial cells, which are actively involved in the production of BM.

Other components occur exclusively in BM and are thought to be produced by the cells which rest upon them. These components, which have been designated "intrinsic components" (1), as well as their most important characteristics are listed in Table 1.

Table 1. Characteristics of intrinsic basement membrane components

Component	Molecular structure	MW	Ref.
Type IV collagen	α1 (IV), α2 (IV) triple helix, rod-like	170-194 kD per chain	22-27
Laminin	three chains, A,B1,B2 cross-like structure, one long arm, three short arms	A 440 kD B1 225 kD B2 205 kD	28-36
Heparan sulphate proteoglycan	heterogeneous: protein core (20%), GAG side chains (80%)	160-750 kD	37-40
Nidogen	rod-like with two globular extensions	150 kD	41-43

Type IV collagen, as other collagens, has a triple helical configuration, formed by two different chains α1 (IV) and α2 (IV) (22), in a hetero-/or homotrimeric configuration. A large globular domain, referred to as the noncollagenous domain-1-(NC1), is localized at the carboxy-terminal of type IV collagen. Disulphide bridges formed between the NC1 domains, lead to dimers of type IV collagen molecules (23). Disulphide bridge formation between the four amino terminal parts (7-S domains) also leads to the formation of anti-parallel overlapping tetramers of type IV collagen (24). In addition to end-to-end binding, lateral associations between type IV collagen molecules are involved in the stabilization of the network (25). Short non-helical parts increase the flexibility but also the susceptibility to proteolytic damage (26). Type IV collagen contains high amounts of carbohydrate side-chains, which in addition to the other glycoprotein components of BM may explain the PAS positive staining reaction of BM in tissue sections. The flexible network formed by type IV collagen molecules is the structural backbone of the BM, to which the other components can bind. Specific binding sites on the type IV collagen molecule have been localized for laminin, heparan sulphate proteoglycan and fibronectin (27).
Laminin is a noncollagenous glycoprotein. The molecule has a cross-like configuration, consisting of a 75 nm long arm and three 35 nm short arms which are connected with disulphide bonds. (28). The end of the short arm is formed by two small globular parts, the end of the long arm by one larger globular part. The laminin molecule contains one A chain (MW 440 kD), one B1 chain (MW 225 kD) and one B2 chain (MW 205 kD). Each chain begins as a short arm, they join in the center and the rest of the chains projects down the long arm (29, 30). Globular domains at the end of the short arms bind to type IV collagen (31). The central part of the laminin molecule interacts with cell surfaces by binding to plasma membrane receptors (32, 33). Binding to heparan sulphate proteoglycan is realized by specific domains at the long arm (34). Immunocytochemical studies at light and electron microscopical level have shown that laminin occurs exclusively in BM, especially in the lamina rara (35). However, other investigators found also localization in the lamina densa (36).
Heparan sulphate proteoglycan (HSPG) consists of a core protein, to which side chains of glycosaminoglycans are bound (37, 38). More than 50% and maybe even 80% of the molecule consists of glycosaminoglycans (38). The core protein has a structure which is unique for BM HSPG (38). The core protein of bovine glomerular BM HSPG (MW 128 kD) has been identified (39). HSPG occurs in the BM as aggregated clusters in the lamina rara (7).It probably plays a role in the attachment of cells to BM and, by means of its anionic properties, in the regulation of glomerular filtration (6, 7). Besides HSPG, other proteoglycans, bearing chondroitin sulphate and dermatan sulphate side chains, have been found in BM (40).
Intact nidogen has a MW of 150 kD, and is highly susceptible to proteolysis (41, 42). The self aggregating tendency and binding to laminin and type IV collagen strongly suggests that nidogen may serve as a bridging molecule within the BM (43).

Immunostaining with antibodies to nidogen was restricted to BM and showed codistribution with laminin (41). In addition to components present in almost all BM, other structural components have been identified, which do not localize in all BM. Some of them are not restricted to BM and have been found in interstitial stroma.
Entactin is a highly sulphated glycoprotein with a MW of 145 kD (44). Although in earlier reviews (1, 45) entactin, was considered to be an intrinsic component, recent studies (21) have shown that it is mainly a product of stromal fibroblasts. Ultrastructural localization of entactin demonstrated variations in localization of entactin within the BM of different rat tissues (46). Evidence has been published which suggests that entactin and nidogen might be identical (42).
Bullous pemphigoid antigen is a glycoprotein (MW 220-240 kD) restricted to stratified squamous epithelium and localized in the lamina rara and or hemidesmosomes (47, 48). The origin of the bullous pemphigoid antigen is still a matter of debate: a BM association is still conceivable but some authors claim that it is an epithelial cell membrane antigen (49).
Fibronectin exists in two forms, the soluble plasma form, and the insoluble tissue form (50). The "V"-shaped molecule is a glycoprotein (MW 440 kD), composed by two polypeptides of 220 kD. Immunolocalization of fibronectin results in interstitial stroma and BM localization (51, 52). One of the main functions of fibronectin is the binding of cells to matrix components (50).
Type V collagen is composed by three distinct αchains, α1(V), α2(V), α3(V), the molecular structure resembles type IV collagen (53). Interstitial stromal localization, as well as close relation to the stromal side of vascular BM has been found (54).
Epidermolysis bullosa acquisita antigen has a native MW of over 800 kD and is composed by a major chain of 290 kD and a minor chain of 145 kD (55, 56). The antigen has been identified as the collagenase insensitive globular domain of type VII collagen (57). This glycoprotein with collagen-like domains probably plays a role in dermal-epidermal adherence (56,57).
Further evidence for the existence of other components can be derived from a study by Hessle et al. (58). These investigators generated monoclonal antibodies against placental membranes and found several BM-specific antibodies which did not react with one of the known components. Another interesting finding was that some antibodies detected epitopes which occurred only on some BM and not on others. Heterogeneity of BM constituents and changes during ontogenesis were observed in human kidneys with monoclonal antibodies to BM components (59).
These data suggest that there may be significant heterogeneity between BM of different cells and tissues. Recently, in our laboratory we raised monoclonal antibodies to BM components by immunizing with amnionic membranes. These antibodies show organ as well as species specificity and lack immunoreactivity to known BM components (60).

1.4 CELL-BINDING TO BASEMENT MEMBRANES

In immuno-electron microscopic studies laminin has been localized very close to the plasma membrane (61). Furthermore, laminin binding proteins (MW 70 kD) have been extracted from tumor cells and normal muscle cells (62-64). Specific laminin receptors on the cell surface and laminin-cell interactions have been extensively reviewed elsewhere (65). Recently, evidence has been found for the existence of two distinct cell-membrane receptors for laminin, one binding to fragment 1 on the short arm and the other to fragment 8 on the long arm (66). A glycoprotein complex, cell substratum attachment (CSAT) antigen (MW 140 kD), which binds to laminin and fibronectin, has been isolated from fibroblasts and myoblasts (67, 68). Amino acid sequences of receptor binding sites have been localized on fibronectin and laminin (69, 70). A special surface associated protein involved in binding of cells to type IV collagen has been found earlier (71).

1.5 BASEMENT MEMBRANES IN CANCER

Studies of BM in cancer have mainly focused on two distinct areas. Firstly, BM have been investigated in relation with tumor invasion and metastasis. Secondly, the production of BM components as a marker of cellular differentiation has been explored.

1.5.1 Basement membranes and invasion

Tumor invasion can be defined as the extension of neoplastic cells beyond the natural borders of the tissue or cell type from which they derive. As a result of this active process of infiltrative growth the neoplastic cells occur in tissue compartments where they do not belong. For epithelial tissues BM are the natural borders. In invasive carcinoma for example, the malignant epithelial cells penetrate the epithelial BM and migrate into the surrounding mesenchymal stroma. Prior to the development of hematogenous metastases the carcinoma cells have to gain access to the vascular lumen. During this process the invasive cells have to penetrate a second - the endothelial - BM. At the site of the development of a hematogenous metastasis the embolized neoplastic cells again traverse the endothelial BM. It appears therefore that in the process of invasion and metastasis the interaction between neoplastic cells and BM plays a pivotal role.
The study of BM morphology in invasive cancer is not entirely new. Early attempts by Ozzello (9) to outline BM in breast cancer by PAS staining, were frustrated by the occurrence of PAS reactive glycoproteins not only in BM but also in interstitial connective tissue. Electron microscopy appeared to be a more suitable technique for the study of BM (72). Several ultrastructural investigations have been published, describing BM or basal lamina morphology in a variety of cancer types. Zelickson

(73) was among the first, reporting on the continuity of the BM around basocellular carcinomas. Other investigators, however, reported discontinuity in BM in connection with this type of neoplasm (74, 75) Gould and coworkers (76-78) published several studies on the BM (which in this context they preferred to call basal lamina) in epithelial neoplasms. Their studies showed a very broad spectrum of patterns of BM deposition in carcinomas. In the breast, for example, BM deposition appeared to parallel myoepithelial differentiation, being notably absent, e.g., in tubular carcinoma (78). Well differentiated squamous cell carcinomas in general retained their ability to deposit a BM but undifferentiated carcinomas showed only scanty BM production (76). Particularly interesting was the finding of BM synthetic capacity in metastatic neoplastic cells as well as the occurrence of interruptions or "gaps" in BM in in situ or borderline lesions (76). In a recent study of squamous cell carcinomas of the lung Dingemans and Mooi (79, 80) showed that in the expanding periphery of the tumor, nests of tumor cells were often surrounded by the BM of the original alveolar epithelium. In addition, isolated single and highly irregular clusters of tumor cells, as well as tumor cells which had invaded bloodvessels, were always surrounded by a basal lamina, which was presumably newly formed.
Taken together these ultrastructural observations show that in some in situ and borderline lesions and in most invasive carcinomas defects in the BM occur. On the other hand carcinoma cells often retain the possibility to deposit BM material, even in metastatic foci. These findings are difficult to reconcile with the conventional concept of passive piercing of the BM by aggressively infiltrating tumor cells.
In recent years immunocytochemical localization of BM components has greatly expanded the possibilities to study BM in cancer. Initial studies in this area, using naturally occurring antibodies against bullous pemphigoid antigen, supported ultrastructural findings of BM defects in basocellular cancer of the skin (81). However, using antibodies to laminin and type IV collagen, continuous BM were found in basal cell carcinoma, whereas bullous pemphigoid antigen showed complete absence (82). Albrechtsen et al. (83) were among the first to show by immunocytochemical staining of defined BM components that in the breast in situ carcinomas, intralobular or intraductal proliferations of neoplastic epithelial cells, are surrounded by an intact BM. In contrast, invasive carcinomas showed BM with focal defects, especially in areas of invasion. Similar findings were reported by Siegal et al.(84). In addition, these investigators found cytoplasmic immunoreactivity for laminin and type IV collagen in primary as well as in metastatic tumor cells. Burtin et al.(85) performed an immunofluorescence study on colonic adenocarcinomas and found weaker and more irregular staining than in normal mucosa, resulting in patchy BM patterns. Subsequent immunocytochemical studies of a variety of carcinomas in different tissues have shown that in the majority of benign and in situ lesions BM can be detected in a normal continuous configuration whereas in the majority of invasive lesions, focal, partial or complete absence of BM components is found. Initial reports focused on continuity of BM in benign neoplasms, discon-

tinuous and irregular basements membranes in in situ lesions and absence of BM in invasive carcinomas (86, 87). However in some benign neoplasms, such as syringomas and pilomatrixomas of the skin (88) and pleomorphic adenomas of the salivary gland (89), BM discontinuities have been found. Conversely, in some malignant neoplasms, such as adenoid cystic carcinomas of the salivary gland (89) and laryngeal squamous cell carcinoma (90, 91), the BM remains intact. A brief summary of the findings in various tumors in different organs is given in Table 2.

Table 2 Basement membrane alterations in benign and malignant human neoplasms.

Organ	Type of neoplasm	Basement membrane morphology	Ref.
Skin	Squamous cell carcinoma	Focal gaps to extensive loss	91,92,93
	Basal cell epithelioma	Intact, continuous	91,92,93
	Benign adnexal tumors	Distinct, continuous In some tumor types discontinuous	88
	Keratoacanthoma	Discontinuities	94
	Malignant adnexal tumors	Discontinuous or lacking	88
Head and Neck	Squamous cell carcinoma		
	Oropharynx	Well developed, focal discontinuities	90,91
	Tongue		90
	Larynx		90,91,95,96
Breast	Fibroadenoma	Intact, continuous	83,84,92
	Sclerosing adenosis	Intact, continuous	83,84,86,87 97
	Adenocarcinoma	Discontinuous or lacking	83,84,86,87 92,97
Colon	Adenoma	Intact, continuous	85,86
	Adenocarcinoma	Discontinuous or lacking	85,86,116
Pancreas	Islet cell adenoma	Intact, continuous	98
	Adenocarcinoma	Irregular, discontinuous	86,98
Prostate	Hyperplasia	Intact, continuous	86
	Adenocarcinoma	Discontinuous or lacking	86
Salivary gland	Pleomorphic adenoma	Poorly developed, discontinuous	89
	Adenoid cystic carcinoma	Intact, continuous	89
	Warthin's tumor	Intact, continuous	89
	Mucoepidermoid tumor	Discontinuous or lacking	89
Uterus	Endometrial adenocarcinoma	Discontinuous or lacking	86,118
	Squamous cell carcinoma of cervix	Intact or focal gaps	92,102
Lung	Adenocarcinoma	Intact, continuous	87,92
	Squamous cell carcinoma	Irregular, discontinuous	87,92

The lack of immunoreactivity for BM components in the majority of invasive carcinomas is in concert with conventional concepts regarding the progression of in situ to invasive lesions.

Invasive tumor cells penetrate the original BM and are then no longer demarcated by this barrier (99). If consistent and reproducible this phenomenon would be very useful for the distinction between pre-invasive (in situ) and invasive lesions. It is quite evident, however, that BM defects are neither a sine qua non for an invasive, malignant lesion nor a specific hallmark of invasion. In well differentiated squamous cell carcinomas and in endometrial adenocarcinomas ultrastructural studies (78) and immunohistochemical studies (100, 101) have shown the consistent presence of BM deposits, observations which are somewhat at variance with previous histochemical studies (8, 86). On the other hand focal BM defects occur in in situ lesions (96). The latter observation is rather intriguing and suggests that prior to morphologically recognizable invasive growth the original BM may be disrupted. In reality, therefore, the situation is quite a bit more complex than the conventional concept of progression from in situ to invasive neoplasia suggests. Intact BM can occasionally be found in invasive lesions and, conversely, in noninvasive lesions BM defects may occur. BM defects even occur in inflammatory conditions when the epithelium is invaded by leucocytes (96, 98, 100-102).

An unresolved problem in this context is that the results of ultrastructural studies are not completely congruent with those of immunocytochemical studies. In several types of cancer by electron microscopy continuous BM were detected whereas immunocytochemical studies in similar lesions showed focal BM defects (8, 76, 79, 80, 86). Several factors should be considered in explaining for these discrepancies. Firstly there may be a sampling problem: immunocytochemistry allows the screening of large areas of tumor tissue whereas by electron microscopy a thin basal lamina may be detected which, especially after tissue processing procedures, might escape immunocytochemical detection. Secondly, differences in the approach of the problem may have played a role: electron microscopists have almost invariably looked for pericellular deposition of BM material (76, 79, 80), whereas immunocytochemists have emphasized the occurrence of BM interruptions (8, 86). It is evident that additional electron-immunocytochemical studies are necessary to resolve this issue. These contrasting observations quite naturally lead to the question of what mechanisms are involved in the generation of BM discontinuities. It should be emphasized here that the BM is not a static structure but is continuously catabolized and redeposited (103). If the idea of mechanical rupture of the BM due to expansive tumor growth is dismissed as being too simplistic, the conclusion remains that BM defects are caused by an imbalance between breakdown and production. This could be due to increased degradation by cancer cells or to decreased synthesis or decreased assembly of BM constituents or a combination of these processes. That actively invasive cancer cells degrade rather than accumulate BM is supported by several observations. Many types of human and murine tumor cells have been shown to degrade BM in vitro (104-108). Liotta et al (104, 105) described a collagenase specific for type IV collagen whose production correlated with the metastatic potential of a series of B16 melanoma sublines. Kramer et al. (106, 107)

identified an endoglycosidase in B16 melanoma cells which selectively degraded heparan sulphate proteoglycan. Human breast carcinoma cells have been shown to contain immunoreactive type IV collagen specific collagenases (109). The degradation of the BM by leukocytes in inflammatory reactions (94, 110, 111) is also supportive of enzymatic catabolism as an important mechanism. The evidence in favor of insufficient synthesis or defective assembly of BM components by cancer cells is scanty. The most important observation in this respect is the occurrence of intracytoplasmic immunoreactivity for laminin and type IV collagen in invasive or metastatic tumor cells, even in the absence of an extracellular formed BM (83, 86, 96). The role of laminin in the attachment of cells to the extracellular matrix suggests that this protein or its cell surface receptor may have an important function in the attachment of circulating tumor cells in the initial phase of the development of a metastasis. Evidence in favor of this suggestion has been reported by Varani et al (112).

1.5.2 Basement membrane components as markers of cellular differentiation

This aspect of BM in cancer has not been extensively studied as yet. In principle, BM deposition can be considered as an indicator for the type of differentiation but can also be correlated with the degree of differentiation of a particular neoplasm. BM deposition is a helpful marker in the classification of soft tissue tumors (113-115). In these studies it was shown that immunoreactivity for laminin and type IV collagen is intense in Schwannomas and neurofibromas, less intense in leiomyomas and leiomyosarcomas but negative in fibrous histiocytomas and fibrosarcomas. These promising observations suggest that BM immunocytochemistry may be a useful method for soft tissue tumor classification but additional studies on a large number of various neoplasms will be necessary to validate this suggestion. For the classification of epithelial neoplasms BM may be less helpful. In a study of malignant adnexal tumors of the skin Kallioinen et al. (88) did not find consistent patterns of BM immunostaining of the different types of adnexal tumors.
Several observations support the idea that BM deposition and the degree of differentiation of a carcinoma may be correlated. Firstly, this idea would be consistent with the role of BM in cytodifferentiation and morphogenesis (5). Secondly, culturing of cells on intact BM resulted in organized cell-cell relations and differentiated cytoskeletal polarity (120, 121). Thirdly, in our own immunohistochemical studies on colorectal neoplasms BM were consistently present in well differentiated carcinomas but completely absent in poorly differentiated carcinomas. Similar observations were reported by other authors (85, 116-119) and, by electron microscopy, by Gould et al. (78). On the other hand, in malignant adnexal tumors of the skin such a correlation was less apparent (88). It is far too early to draw any conclusions but the available information warrants further studies on the significance of BM deposition as a differentiation marker in neoplasia.

1.6 AIM OF THE STUDY

From the previous paragraphs it can be concluded that BM play an important role in tumor cell-extracellular matrix interactions, especially with regard to invasion and metastasis. Reliable and reproducible visualization of specific BM components by microscopic histochemical techniques is one of the essential tools to the study of the role of BM in tumor cell-extracellular matrix interactions. To attain that goal specific antibodies to BM had to be raised and characterized. Chapter 2 describes the production of two monoclonal antibodies and a polyclonal antiserum to the human type IV collagen, and their immunohistochemical application. In chapter 3, BM deposition of human epithelial tumor cell-lines is studied in vitro and in xenografts of these tumor cells in nude mice and rats. Because of the human specificity of the monoclonal antibodies to type IV collagen, and the cross-species reactivity of the polyclonal antiserum, it became possible to speculate on the origin of type IV collagen deposited in BM at the tumor-stroma interface in xenografts. Using the obtained antibodies to type IV collagen on suitable treated routinely processed human tumor tissues, as described in chapter 2, a retrospective study of patterns of BM deposition in a large number of colorectal carcinomas could be performed. Chapter 4 deals with the relation between the patterns of BM deposition and prognosis in colorectal cancer, which is largely determined by the capacity of the tumor to invade the vascular system and produce liver metastasis. Applicability of the antibodies to routinely processed tissues and the availability of a computerized data base, containing clinicopathological as well as survival data, strongly facilitated this study. In the literature many discrepancies exist between ultrastructural (morphological) observations and light microscopic (immunohistochemical) observations on BM occurrence in neoplasia. These discrepancies prompted us to perform a comparative study on BM patterns in squamous cell carcinomas of the lung by electron microscopy and immunohistochemistry. In chapter 5, this study is reported with special emphasis on the differences between tumor center and tumor periphery, which appeared to show remarkable differences with interesting biological implications. Nevo-melanocytic lesions often generate serious diagnostic problems. The only two published studies dealing with immunohistochemical detection of BM in nevo-melanocytic lesions showed remarkable discrepancies of the results concerning BM deposition at the border of nevo-melanocytic cells and the adjacent stroma. In chapter 6 we describe BM patterns in benign and malignant nevo-melanocytic tumors and critically discuss the potentials for application of BM immunohistochemistry in diagnostic immunohistopathology. Finally, in chapter 7, the results of these studies concerning BM deposition in neoplastic lesions are discussed against the background of the recent literature concerning tumor cell-extracellular matrix interactions, and future prospects are speculated upon.

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CHAPTER 2

Human specific anti-type IV collagen monoclonal antibodies, characterization and immunohistochemical application.*

2.1 INTRODUCTION

Extracellular matrix consists of basement membranes (BM) and interstitial stroma. BM separate epithelia from the stroma and surround individual cells such as adipocytes, muscle cells and Schwann cells (1). BM contribute to the tissue structure, they play a role in cell differentiation, they form semipermeable filtration barriers and they provide scaffolds for tissue repair and regeneration (2, 3). All BM contain type IV collagen, laminin and proteoglycans. The type IV collagen molecule is a discontinuous triple helix of about 400 nm length, composed by two distinct chains α1 (IV) and α2 (IV) (4, 5). At the N-terminal region (7S-domain) four type IV collagen molecules are cross-linked and form tetramers. The C-terminal noncollagenous domain (NC1), binds with the NC1 domain of another tetramer (6). This supramolecular organization provides a regular flexible network and forms the structural backbone to which other components such as laminin and heparan sulphate proteoglycan can bind (7).

Immunohistochemistry has proven to be a valuable tool for the study of the BM under various physiological and pathological conditions.

Immunohistochemical studies of type IV collagen have yielded important information in regard of the role of BM in developmental biology (8). Monoclonal antibodies, especially human specific antibodies, in combination with cross species reactive polyclonal antisera to type IV collagen have been helpful in studies concerning the origin of this BM component in xenografts of normal and neoplastic human tissues in nude mice (9-11). Furthermore antibodies to BM components have been used in the study of neoplastic disease in diagnostic pathology (12-15). We have developed two new monoclonal antibodies specific for human type IV collagen epitopes in frozen as well as routinely fixed and processed tissue sections. The present paper describes these antibodies and provides optimal procedures for their immunohistochemical application.

* Havenith MG, Cleutjens JPM, Beek C, v.d. Linden E, De Goeij AFPM, Bosman FT.
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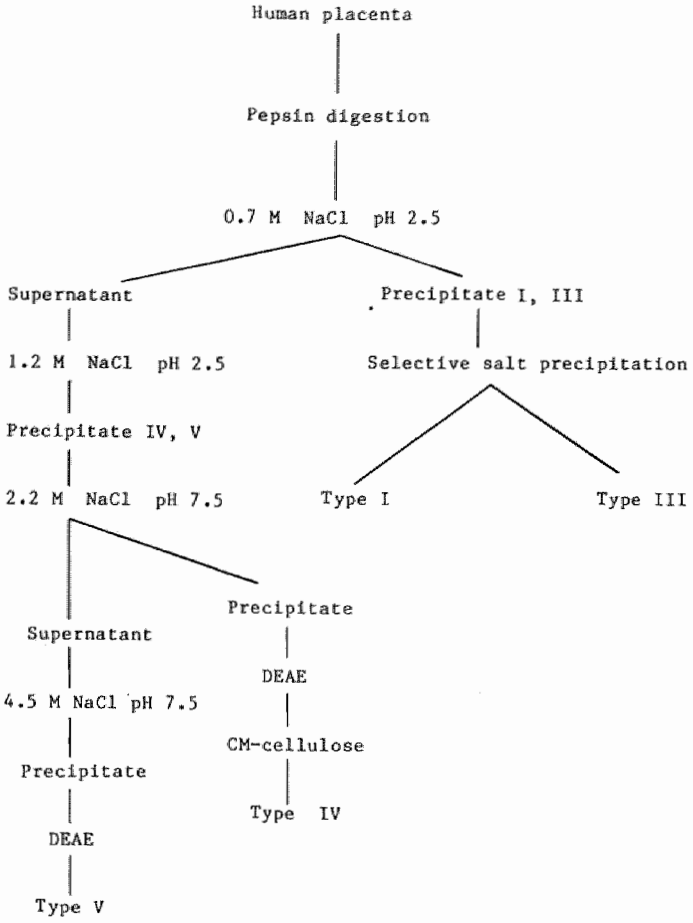

Figure 1

2.2 MATERIAL AND METHODS

2.2.1 Isolation and characterization of collagens

Various collagen types were isolated from human placenta by limited pepsin digestion, fractionated by salt precipitation and chromatographic techniques modified from those of Miller & Rhodes (16) as schematically represented in Fig. 1.
The purity of the isolates was analyzed by sodium dodecyl sulphate-5% polyacrylamide gel electrophoresis (17), followed by blotting onto nitrocellulose paper (18) and staining with aurodye (Janssen Life Science Products).

2.2.2 Preparation and isolation of hybridomas

Female balb/c mice (TNO Zeist) were immunized with human placental type IV collagen (Sigma). The first injection (200 μg in complete Freunds adjuvant) was administered intraperitoneally. Booster injections of 100 μg of type IV collagen (in incomplete Freunds adjuvant) were given intraperitoneally at three week intervals. At the fourth day after the last injection, spleen cells were isolated and fused in 50% polyethylene glycol with Sp2/0 myeloma cells, grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum. The cells were initially cultured in HAT medium to select for hybridomas and antibody production was detected by ELISA. To this end wells of a microtiter plate (polypropylene) were coated with 50 μl of a solution of 5 μg/ml type IV collagen in 0.1 M carbonate buffer pH 9.6. Supernatants of the tested wells were incubated overnight, followed by peroxidase labelled rabbit antimouse IgG antibodies. Peroxidase activity was detected by adding O-phenylene diamine substrate. Selected hybridomas were cloned by limiting dilution and expanded. Ascites was produced by intraperitoneal injection of hybridoma cells in Balb/c mice.

2.2.3 Preparation of polyclonal antiserum

Two rabbits were immunized intracutaneously with a solution of 1 mg/ml type IV collagen suspended in an equal volume of complete Freunds adjuvant. After two weeks a booster injection was given, followed by three more booster injections at two weekly intervals with the antigen in incomplete Freunds adjuvant.

2.2.4 Immunological characterization

Immunoreactivity of the monoclonal antibodies and the polyclonal antisera was tested by ELISA. Microtiter plates were coated with type I, III, IV and V collagen and the assay was carried out as described above.
Immunospecificity of the antibodies was tested by immunoblotting. SDS-polyacrylamide gel electrophoresis of beta-mercaptoethanol reduced and non-reduced type I and IV collagen were blotted onto nitrocellulose-paper. Immunoreactivity was visualized by peroxidase labelled rabbit anti mouse Ig antibodies and diaminobenzidine as substrate. Isotyping of the monoclonal antibodies was performed in ELISA by coating microtiter plates with monoclonal antibody followed by isotype specific goat anti mouse IgG1, IgG2A, IgG2B and IgM. Finally the wells were incubated with peroxidase labelled rabbit anti goat Ig antibodies.

2.2.5 Tissues

Samples of various human tissues were fixed in 4% neutral buffered formaldehyde, processed routinely and embedded in paraplast and sectioned at 4 μm. Tissue samples were also snap frozen in isopentane, quenched in liquid nitrogen. Fresh tissue samples were also collected from rat, mouse, rabbit, cow and dog and snap frozen. Frozen tissues were cryostat sectioned at 4 μm.

2.2.6 Enzymes

Immunoreactivity could only be demonstrated in paraffin sections through enzymatic digestion after deparaffinization and rehydration. To obtain an optimal procedure various enzymes were tested under varying conditions as summarized in Table 1.

Table 1

Enzyme	Range of concentration	Range of buffer condition	Range of incubation time [hours]	Temperature
Pepsin (Boehringer)	0.1%–0.5%	HAc or HCl pH 1.0–3.0	$^1/_2$–2	RT/37 °C
Trypsin (Difco)	0.1%–0.5%	50 m*M Tris*-HCl pH 7.0–8.5 (adj. 2 *N* HCl) $CaCl_2$ 0.1–0.5%	$^1/_4$–1	RT/37 °C
Pronase (Boehringer)	0.01%–0.05%	50 m*M Tris*-HCl pH 7.5–8.0 (adj. 2 *N* HCl)	$^1/_4$–1	RT/37 °C
Papaïn (Boehringer)	0.1%–0.3%	50 m*M* NaAc buffer 0.2 *M* NaCl pH 5.0–6.0	$^1/_4$–1	RT/37 °C
Hyaluronidase (Cooper Biomedical)	0.1%–0.5%	0.1 *M* sodium phosphate buffer 0.15 *M* NaCl pH 4.5–6.0	$^1/_2$–2	RT/37 °C

2.2.7 Immunohistochemistry

Frozen sections were fixed 20 min in acetone (-20°C) and endogenous peroxidase activity was blocked in 0.3% H_2O_2 in methanol, 20 min, RT. After washing with PBS (3 x 5 min) monoclonal antibodies, undiluted supernatant or 1:400 diluted ascites (diluted in PBS with 1% BSA), were incubated (1hr at RT) in a moist chamber. After washing with PBS the sections were incubated with peroxidase labelled rabbit anti mouse Ig antibodies (1hr, RT). After final washing with PBS, a diaminobenzidine-H_2O_2 substrate was used to visualize the immunoreactivity.
Paraffin sections were deparaffinized, rehydrated and blocked for endogenous

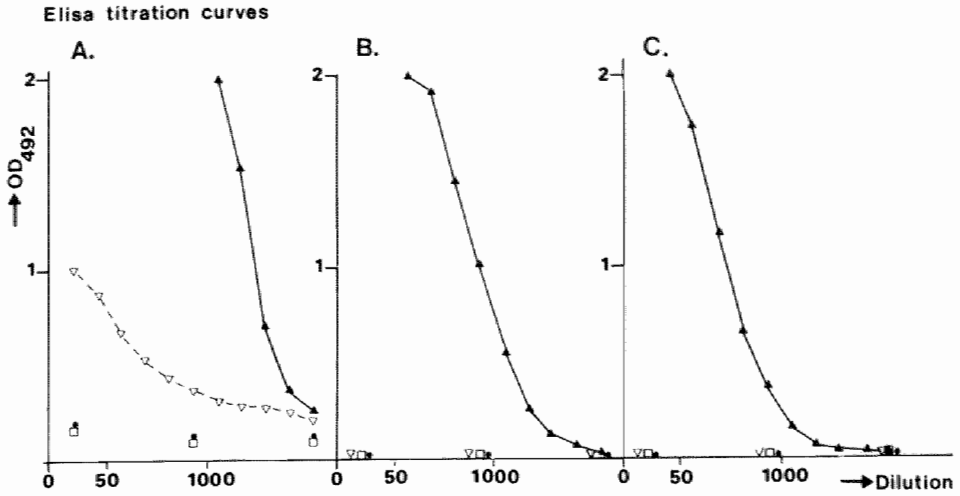

Fig. 2 Wells coated with:
- ▲ human type IV collagen
- ▼ human type V collagen
- • human type I collagen
- □ human type III collagen

Titrated with:
A. polyclonal antiserum to type IV collagen
B. monoclonal antibodies to type IV collagen (1042)
C. monoclonal antibodies to type IV collagen (1043)

peroxidase, followed by enzyme digestion, before applying the monoclonal antibodies. The further procedures were as for frozen sections.

2.3 RESULTS

2.3.1 Polyclonal antiserum

In the ELISA the polyclonal anti type IV collagen antiserum showed no cross-reactivity with collagen types I and III and weak reactivity with type V collagen (Fig. 2). In immunoelectroblotting experiments (Fig. 3) the polyclonal antiserum showed immunoreactivity with the various reduced and nonreduced type IV collagen bands, confirming its specificity. Immunohistochemical staining on frozen as well as on pepsin treated paraffin sections revealed a BM specific staining pattern. In tissue sections interstitial collagens were not detected nor could the BM specific staining be blocked by type V collagen, indicating the type IV collagen specificity of the immunoreactivity. On formalin fixed paraffin embedded sections immunoreactivity was only obtained after preincubation of the sections with pepsin or pronase (Fig.

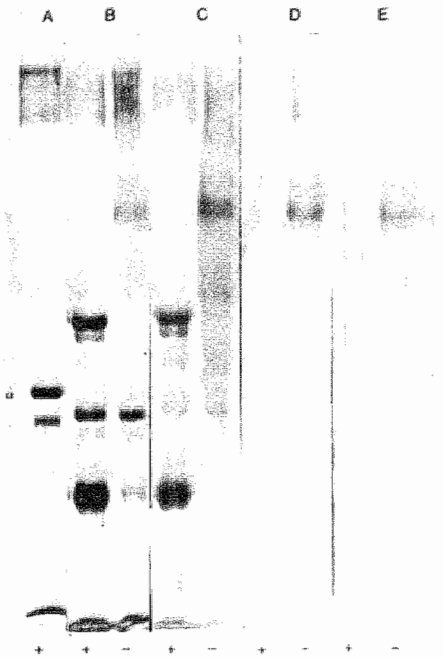

Fig. 3 A. human type I collagen stained with aurodye (lane 1)
B. human type IV collagen stained with aurodye (lane 2 and 3)
C. human type IV collagen reacted with polyclonal anti type IV collagen antiserum (lane 4 and 5)
D. human type IV collagen reacted with monoclonal anti type IV collagen antibodies, 1042 (lane 6 and 7)
E. human type IV collagen reacted with monoclonal anti type IV collagen antibodies, 1043 (lane 8 and 9)
\+ indicates reduction with β-mercaptoethanol
− no reduction
α indicates the 100 kD band of heat denaturated α1(I)

4 A and B) or with papain. This staining protocol was compatible with other fixation with formalin, Bouins', periodate-lysine-paraformaldehyde and picric-acid paraformaldehyde. Optimal conditions for pepsin treatment were 0.1% pepsin in 0.1N HCl, 30 min at RT, for pronase 0,04% (w/v), in 50 mM Tris-HCl adjusted (with 2 N HCl) to pH 8.0, 30 min at RT and for papain 0.2% (w/v) in 50 mM NaAc buffer with 0.2 M NaCl pH 5.5, 30 min RT (Table 2).

2.3.2 Monoclonal antibodies

Initial screening of the cloned hybridomas by ELISA yielded two type IV collagen reactive clones (1042 and 1043). Isotype determination showed that the monoclonal

Table 2. Influence of pretreatment conditions on type IV collagen immunoreactivity

Pronase

	pH		Concentration [% w/v]			Time [hours]				Temperature	
	7.5	8.0	0.03	0.04	0.05	1/4	1/2	3/4	1	Room-temp.	37 °C
p.c. 1042, 1043	±	+	±	+	±	±	+	±	−		+

Papain

	pH			Concentration [% w/v]			Time [hours]				Temperature	
	5.0	5.5	6.0	0.1	0.2	0.3	1/2	1	1 1/2	2	Room-temp.	37 °C
p.c. 1042, 1043	±	+	±	±	+	±	+	±	−	−	+	

p.c.-polyclonal antiserum; 1042, 1043-monoclonal antibodies; + strong, ± weak, − no reactivity

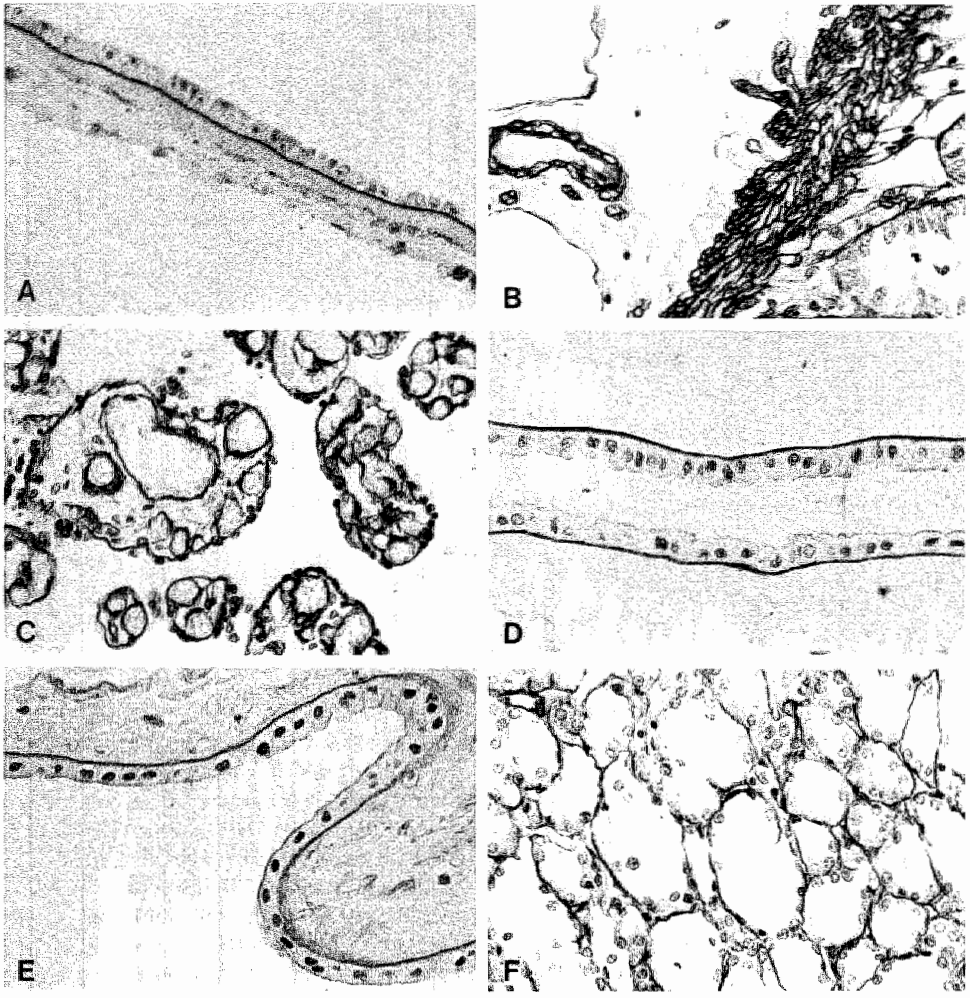

Fig. 4 Polyclonal antiserum to human type IV collagen (immunoperoxidase) applied to A human amnion (pepsin, 220 x) and B normal human colon (pronase, 340x). Monoclonal antibody (1042) to human type IV collagen (immunoperoxidase) applied to C human placenta (pronase, 340 x) and D human amnion (papain, 340x). Monoclonal antibody (1043) to human type IV collagen (immunoperoxidase) applied to E human amnion (pronase, 340 x) and F human thyroid gland (pronase, 340 x).

antibody 1042 is an IgG2B and 1043 an IgG1. Immunospecificity testing by ELISA to collagen types I, III, IV and V confirmed that these monoclonal antibodies reacted only with type IV collagen (Fig. 2 B and C).
In immunoblotting studies immunoreactivity could be detected in the high molecular weight region of non-reduced type IV collagen with both monoclonal antibodies. Reduced type IV collagen showed only one very weak band in the low molecular weight region with both monoclonal antibodies (Fig. 3 D and E). No reactivity was found with other collagen types.
Immunohistochemical staining of frozen sections resulted in BM immunoreactivity, identical to the pattern obtained with the polyclonal antiserum. On formalin fixed paraffin embedded sections immunoreactivity was only obtained after enzyme pretreatment. Of the various enzymes tested only pronase and papain gave consistent results (Fig. 4 C-F). The effect of various conditions on the obtained immunoreactivity is summarized in table 2. Optimal conditions for pronase treatment were 0.04% (w/v) of pronase in 50 mM Tris-HCl adjusted (with 2 N HCl) to pH 8.0, 30 min at RT and for papain 0.2% (w/v) in 50 mMNaAc buffer with 0.2 M NaCl pH 5.5, 30 min RT.

2.3.3 Species and organ specificity

Human, rat, mouse, rabbit, bovine and canine tissues were studied for their BM immunoreactivity with the monoclonal antibodies 1042 and 1043. On frozen sections as well as paraffin sections immunoreactivity was obtained with human tissues only, the only exception being reactivity of 1042 with BM in rabbit tissues. Testing on an extensive panel of various human tissues showed immunoreactivity only with BM. Reactivity with interstitial collagens was notably absent. All epithelia expressed immunoreactivity to type IV collagen at the epithelial-stromal interface. BM surrounded individual cells such as adipocytes, smooth and striated muscle, Schwann, and decidual cells and were demonstrated adjacent to lining cells such as endothelium, mesothelium, amnion and ependyma.

2.4 DISCUSSION

The BM has become a structure of high interest in relation to its role in normal development (19), neoplasia (20), and its function as a filtering barrier e.g. in the glomerulus (21). Immunocytochemistry has proven to be a valuable tool to study BM, essential requirements being specific antibodies and reliable tissue processing procedures. The present report addresses both aspects of BM immunocytochemistry.
Polyclonal antisera to type IV collagen from various sources have been available for some time now and conditions for reliable immunocytochemical application on routinely processed tissues have been worked out by Barsky et al. (22). Our results

largely confirm these findings. We found that in addition to pepsin, pronase and papain could also be used to obtain immunoreactivity in paraffin sections of tissues fixed in various fixatives.

The first monoclonal antibody to human type IV collagen,which was cross species reactive, was described by Sundarraj andWilson (23). Sakai et al. (24) produced a human specific monoclonal antibody to type IV collagen. None of these antibodies showed organ specificity. Foellmer et al. (25) raised two monoclonal antibodies to type IV collagen, one human specific and one cross-species reactive. Odermatt et al. (26) described two monoclonal antibodies, one of these showing immunoreactivity only with native human and bovine type IV collagen and the other being entirely human specific. These monoclonal antibodies showed some tissue specificity because neither the corneal BM nor Descemet's membrane reacted with the antibody. Scheinman and Tsai (27) also reported on a cross-species reactive monoclonal antibody. Only the mesangium and the subendothelial plane of the glomerular BM, the lens capsule and cornea were stained, indicating some degree of organ specificity. Our monoclonal antibodies 1042 and 1043 appeared to be almost completely human specific, the only exception being reactivity of 1042 with rabbit tissues. Organ specificity could not be detected, all the examined tissues showed immunoreactivity at the expected BM localization. An important finding was that both monoclonal antibodies could be applied to formalin fixed paraffin embedded tissue sections after preincubation with pronase or papain. Pepsin treatment, as described by Barsky et al. (22) was not effective. For all monoclonal antibodies the optimal tissue treatment protocol will depend on the characteristics of the involved epitope, which call for a specific protocol for each monoclonal antibody.

From our results and the data on anti human type IV monoclonal antibodies in the literature, we can conclude that although the basic structure of type IV collagen is rather similar in all species (as evidenced by the lack of species specificity of polyclonal antibodies) species specific epitopes occur. Species specific antibodies can be rather useful e.g. in cancer xenografting experiments in which these reagents may be used to detect the origin of BM (9-11). A similar conclusion can be drawn with regard to tissue- or organ specific epitopes. Although these appear to occur, it is likely that BM heterogeneity is more related to variations in heparan sulphate proteoglycan content and composition, and to the occurrence of organ specific components, of which the bullous pemphigoid antigen in epidermal BM might be an example (28). Others, however have claimed that the bullous pemphigoid antigen is more likely a cell membrane antigen than a BM antigen (29).

In conclusion, our results show that type IV collagen immunoreactivity can be reliably detected in a variety of routinely processed tissues with polyclonal as well as monoclonal antibodies, provided that appropriate enzymatic treatment is applied. The lack of tissue and organ specificity make the described monoclonal antibodies especially suitable for diagnostic use in histopathology. The species specificity is a rather useful characteristic in human-nude mouse xenotransplantation studies.

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CHAPTER 3

Origin of basement membrane type IV collagen in xenografted human epithelial tumor cell-lines. *

3.1 INTRODUCTION

Basement membranes (BM), which connect epithelia with the underlying stroma, are composed of macromolecules such as type IV collagen (1), laminin (2, 3) and heparan sulphate proteoglycan (4). Using specific antibodies to one or more of these constituents it has been shown that in many carcinomas a discontinuous BM is deposited at the epithelial-stromal interface. It has been suggested that the ability of cancer cells to generate a BM may be associated with invasive and metastatic behavior (5-10) and may be correlated with patient survival (11). It is generally assumed that epithelial BM are at least partly a product of the epithelial cells. Several investigators have demonstrated that in vitro epithelial tumor cells may continue to produce BM components. Experimental data concerning BM origin in neoplasms in vivo is very scanty. Recently, Damjanov et al. (12) studied the extracellular matrix of human tumor xenografts in nude mice using monoclonal anti-laminin antibodies, exclusively reactive with human laminin, and polyclonal anti-laminin antibodies reacting with human as well as with murine laminin. In many of their xenografts, tumoral BM laminin appeared to be of human as well as murine origin. They concluded that laminin in the extracellular matrix of xenografted tumors is at least partly of stromal origin. In this report we describe a similar study with a cross-species reactive polyclonal anti-type IV collagen antiserum and two human specific type IV collagen reactive monoclonal antibodies in normal and neoplastic human epithelial cells in vivo and in vitro.

3.2 MATERIAL AND METHODS

3.2.1 Tissues

Human cell-lines derived from a colonic adenocarcinoma (5583-S), from transformed amnionic epithelium (WISH), and from an oral epidermoid carcinoma (KB) were used for this study. The characteristics of the 5583-S cell-line are described extensively elsewhere (13). The WISH and KB cell-lines were obtained from the American Type Culture Collection (ATCC), the characteristics of these cell lines are described by Hayflick and Eagle (14, 15). Cells were cultured in Dulbecco's modi-

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Submitted to Virchows Arch (Cell Pathol)

fied Eagle's medium (DMEM, Flow Laboratories) supplemented with 10% fetal calf serum (FCS,Sera Lab).
Cell culturing in collagen lattices was performed under conditions described by Nusgens (16). Instead of rat type I and III collagen, human type I and III collagen isolated from placenta were used as described elsewhere (17). For histological evaluation the collagen lattices were processed as described for the tissue samples.
All tumor cell lines were xenografted in nude mice (NMRI nu/nu supplied by Zentral Institut für Versuchstiere, Hannover, FRG) and nude rats (CD 1 nu/nu supplied by Charles River, Sulzfeld, FRG) by injecting tumor cell suspensions (1.5 x 10^6 cells) subcutaneously. After a period of 2-6 weeks solid tumors of 0.5-1 cm in diameter were obtained.
Small fragments of normal colonic mucosa, including muscularis mucosae, were carefully removed from colonic carcinoma resection specimens and xenografted into nude mice (NMRI nu/nu) as described extensively elsewhere (18). Xenografts were removed after 14 days. Samples of the primary tumor of cell-line 5583-S, of the xenografts and of the collagen lattice cultures were snap frozen in isopentane quenched in liquid nitrogen and stored at 70°C and/or fixed in 10% neutral buffered formalin and further embedded in paraplast.

3.2.2 Antibodies

Details of the preparation and characterization of the polyclonal and monoclonal antibodies have been described extensively elsewhere (17). Briefly, polyclonal antibodies to type IV collagen were raised in rabbits by immunizing with human placental type IV collagen. The titer of the antiserum was monitored by solid phase enzyme immunoassay (ELISA) and the specificity of the antiserum was analyzed by SDS-PAGE followed by immunoblotting as well as staining of various tissues. Monoclonal antibodies to type IV collagen were raised by immunizing Balb/c mice with human type IV collagen. Three days after the final immunization mice were sacrificed and spleen cells were fused with Sp2/O cells. Supernatants were tested for type IV collagen immunoreactivity by ELISA. Cross-reactivity with interstitial collagen (type I and III), type V collagen and laminin was excluded by ELISA. Two type IV collagen reactive hybridomas (1042 and 1043) were obtained and cloned by limiting dilution until monoclonal. Species specificity of these monoclonal antibodies was tested by immunohistochemistry on tissue sections of human as well as various murine tissues.

3.2.3 Immunoblotting of culture supernatants

Supernatants of 5583-S, WISH and KB cells were precipitated with 4M NaCl, dissolved in and dialyzed against SDS-sample buffer and run on a 5% SDS-polyacrylamide gel (19), followed by blotting onto nitrocellulose membranes (20).

3.2.4 Immunohistochemistry

In order to expose the antigenic sites, paraffin sections of tissues were pretreated with pronase (Boehringer, 400 μg/ml in 50 mM TRIS-HCl buffer, pH 8.0, for 30 minutes at 37°C) when monoclonal antibodies were applied, or with pepsin (Boehringer, 0.1 mg/ml in 0.01 N HCl, 30 minutes at 37°C) for the polyclonal antiserum. On frozen sections the antibodies could be employed without enzymatic pretreatment. Sections were incubated for one hour with the primary antibodies and subsequently with peroxidase-labeled rabbit anti-mouse Ig or peroxidase labeled goat anti-rabbit Ig (Dakopatts, Copenhagen, Denmark) for the monoclonal and polyclonal antibodies respectively. After each incubation, sections were washed with PBS (3 x 5 min). Immunoreactive sites were visualized with diaminobenzidine. Finally, the sections were lightly counterstained with Mayer's haematoxylin.

3.3 RESULTS

3.3.1 Antibody specificity

By ELISA the polyclonal anti-type IV collagen antiserum did not react with collagen types I and III and showed only a slight reaction with type V collagen (Fig. 1A). Under immunohistochemistry conditions, however, the antiserum only detected type IV collagen, as indicated by solid phase immunoadsorption of the antiserum with type V collagen. Reactivity with type I, III and V could not be detected with the monoclonal antibodies (Fig.1 B and C). In immunoelectroblotting experiments the polyclonal antiserum expressed immunoreactivity with several reduced and non reduced type IV collagen bands, in the same pattern as the aurodye stain of the reduced and non reduced type IV collagen (Fig. 2 lanes 2-5). However, both monoclonal antibodies showed identical immunoreactivity with a single band in the high molecular region of non-reduced type IV collagen and very weak immunoreactivity with a single band in the low molecular region (Fig. 2 lanes 6-9).
Immunohistochemical experiments further substantiated the specificity of these antibodies. On a wide variety of human tissues both polyclonal and monoclonal anti-type IV collagen antibodies reacted exclusively with BM in an identical pattern. On sections of normal tissues from various other species (including Balb/c and NMRI nu/nu mice) polyclonal anti-type IV collagen antibodies showed a similar pattern of immunoreactivity. The two monoclonal antibodies, however, did not show any reactivity with tissues from these species.

3.3.2 Type IV collagen labelling in primary colonic carcinomas and cancer cell lines

In the primary tumor, from which the 5583-S cell-line was derived, polyclonal as well as monoclonal antibodies localized in BM, which were deposited extensively, but

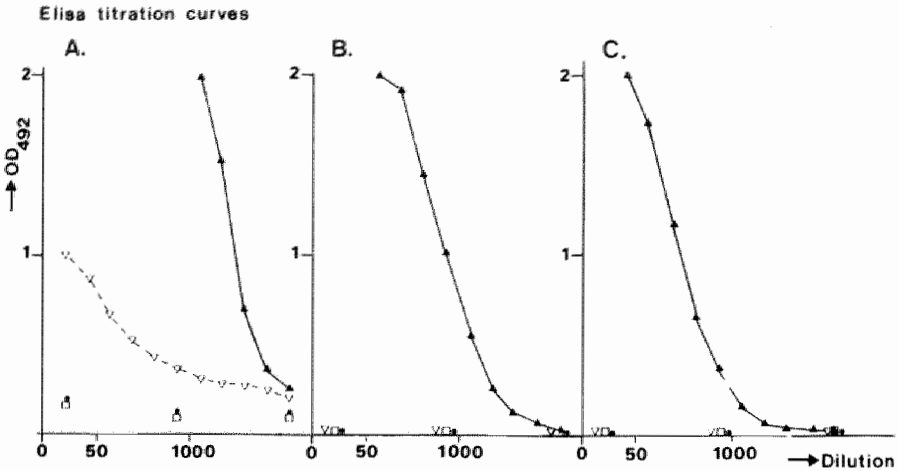

Fig. 1. ELISA testing of reactivity of anti-type IV collagen antibodies with

- ▲ human type I collagen
- ▼ human type III collagen
- • human type IV collagen
- □ human type V collagen

A. rabbit polyclonal anti type IV antiserum
B. 1042, mouse monoclonal anti human type IV collagen antibody
C. 1043, mouse monoclonal anti human type IV collagen antibody

discontinuously and irregularly at the epithelial-stromal interface (Fig. 3). Immunohistochemical studies of 5583-S cells grown in artificial collagen lattices did not reveal any reactivity with the polyclonal or with the monoclonal anti-type IV collagen antibodies (Fig. 4).

Immunoblots of the precipitated tissue culture supernatants of 5583-S cell-line lacked any immunoreactivity with antibodies to type IV collagen, whereas pro α1- and pro α2-chains of type IV collagen were detected in supernatants of WISH and KB cell-lines by this method (data not shown).

WISH and KB cells cultured in collagen lattices showed extensive granular intracytoplasmic and/or pericellular immunoreactivity for type IV collagen with polyclonal as well as monoclonal antibodies, BM like structures could not be identified (Fig 5).

3.3.3 Type IV collagen labelling in xenografts

In xenografts of normal human colonic mucosa polyclonal anti type IV collagen antibodies reacted with epithelial as well as endothelial and smooth muscle BM. All BM were highly regular and continuous. A similar pattern of immunoreactivity was seen with monoclonal anti-type IV collagen antibodies.

In xenografts of 5583-S, WISH, and KB cells immunohistochemical studies with

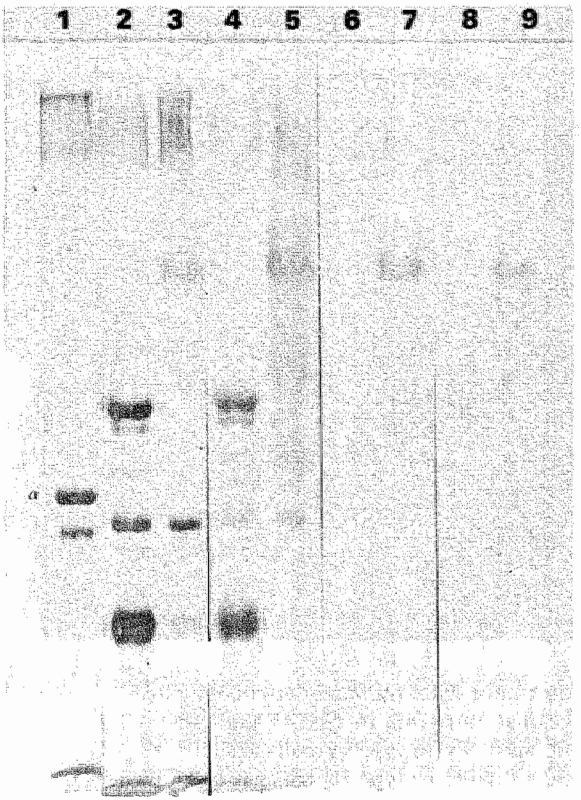

Fig. 2. Western blot of SDS-PAGE separated collagens.

Lane 1	Reduced human type I collagen stained with Aurodye
Lane 2 and 3	Reduced and non-reduced human type IV collagen stained with Aurodye
Lane 4 and 5	Reduced and non-reduced human type IV collagen reacted with polyclonal anti-type IV collagen
Lane 6 and 7	Reduced and non-reduced human type IV collagen reacted with monoclonal 1042
Lane 8 and 9	Reduced and non-reduced human type IV collagen reacted with monoclonal 1043 heat denatured alpha-1 (I), 100 kD.

polyclonal anti-type IV collagen antibody showed reactivity with epithelial as well as vascular BM. The epithelial BM were irregular and discontinuous, many tumor cell nests lacking a BM altogether (Fig. 6A). In 5583-S xenografts, however, both monoclonal antibodies (Fig. 6 B and C) did not show any reactivity for type IV collagen. In WISH and KB xenografts BM like reactivity for type IV collagen with the monoclonal antibodies could be demonstrated clearly (Fig. 7 A and B). BM of vascular structures in the xenografts, however, did not react with the monoclonal anti-type IV collagen antibodies.

A B C

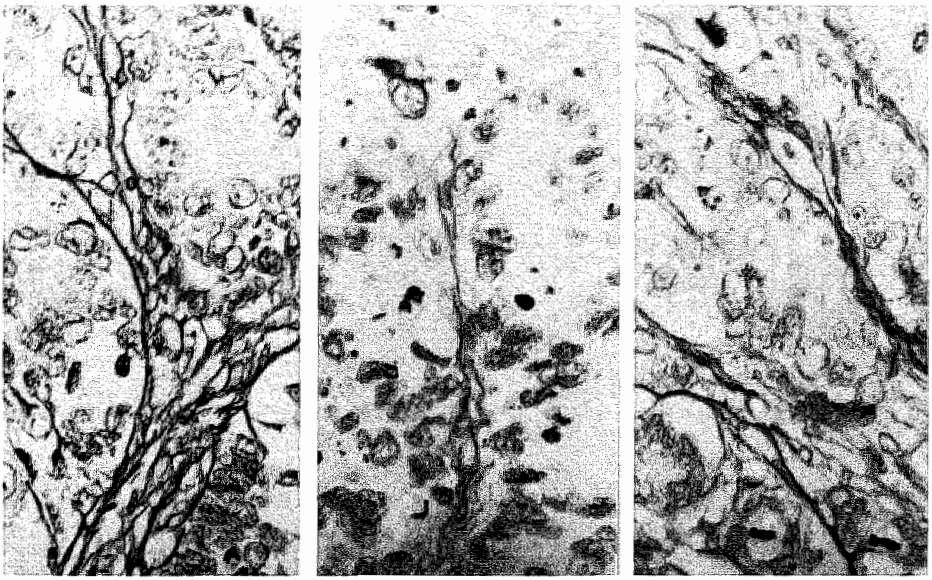

Fig. 3. Paraffin sections of the primary tumor 2983 (immunoperoxidase 340x)
A. cross species reactive polyclonal antiserum to type IV collagen
B. monoclonal human specific antibody to type IV collagen (1042)
C. monoclonal human specific antibody to type IV collagen (1043)
All sections show type IV collagen immunoreactivity at the epithelial-stromal border and perivascularly.

3.4 DISCUSSION

Xenografts of human tissues in nude mice offer an experimental model to study the in vivo interaction of normal or neoplastic epithelial cells with mesenchymal stromal elements. When antibodies which discriminate between human and murine BM components are employed in immunohistochemical studies, the host or graft origin of these components can be determined. In that model Holmstrup (21) examined the presence of laminin and type IV collagen in BM of normal human oral mucosa, transplanted into nude mice. In this study polyclonal antibodies against laminin and type IV collagen reacted equally well with vascular and epithelial BM whereas human specific monoclonal antibodies reacted primarily with vascular BM and only weakly with epithelial BM. Holmstrup concluded that under these conditions oral mucosal cells are able to participate in the formation of BM at the epithelial/stromal interface and that the vessel supply of the transplants is of both human and murine origin. Demarchez et al (22) studied revascularization of human skin transplants onto nude mice, applying murine and human specific antibodies to type IV collagen

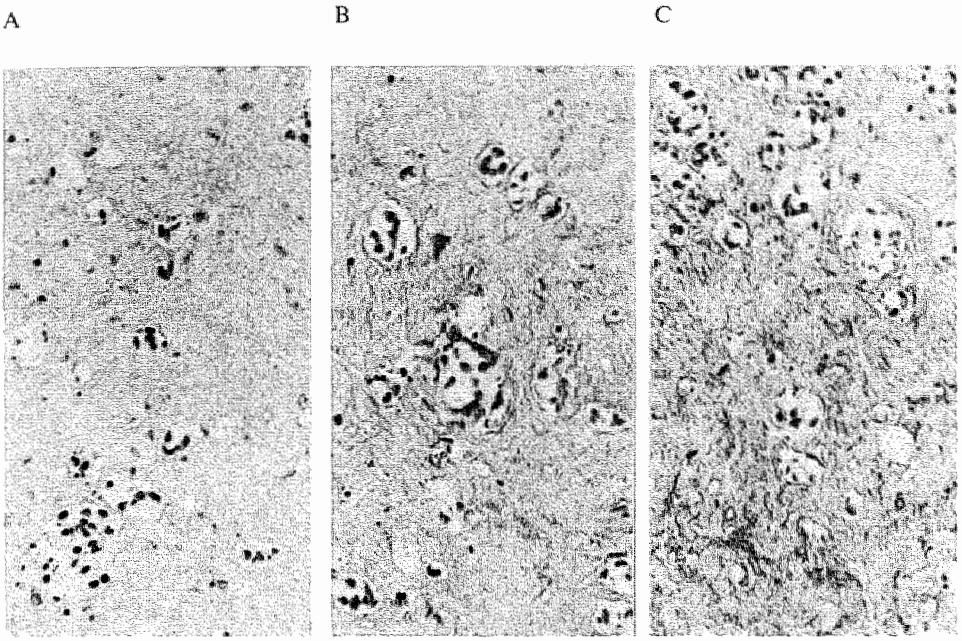

Fig. 4. Paraffin sections of tumor cell-line 5583 cultured in artificial collagen lattices (immunoperoxidase 225x)
A. cross species reactive polyclonal antiserum to type IV collagen
B. monoclonal human specific antibody to type IV collagen (1042)
C. monoclonal human specific antibody to type IV collagen (1043)
No immunoreactivity to type IV collagen is apparent

and HLA-DR antigens for identification of the origin of the BM and endothelium. A shift from human to murine endothelium and a subsequent change from human to mouse type IV collagen in the subendothelium BM was found. Kühl et al found in a coculture of mouse myoblasts with chicken fibroblasts deposition of type IV collagen of chicken origin in the BM around mouse myoblasts (23). Damjanov et al. (12) performed experiments with nude mice xenografts of a lung adenocarcinoma and a yolk sac carcinoma, known to produce cell associated laminin in vitro and a hepatoblastoma and a hepatocellular carcinoma, lacking this characteristic. Using cross-species reactive as well as human specific anti-laminin antibodies the former xenografts were shown to contain pericellular laminin of both human and murine origin, whereas the latter xenografts only demonstrated pericellular laminin of mouse origin. Vascular BM only contained laminin of murine origin.

From these findings it can be concluded that BM in epithelial as well as non epithelial tissues are not exclusively the product of the adjacent cell. Connective tissue elements most likely also contribute to the deposition of the BM. Against this background we studied the origin of BM in human tumor cell-lines, xenografted into

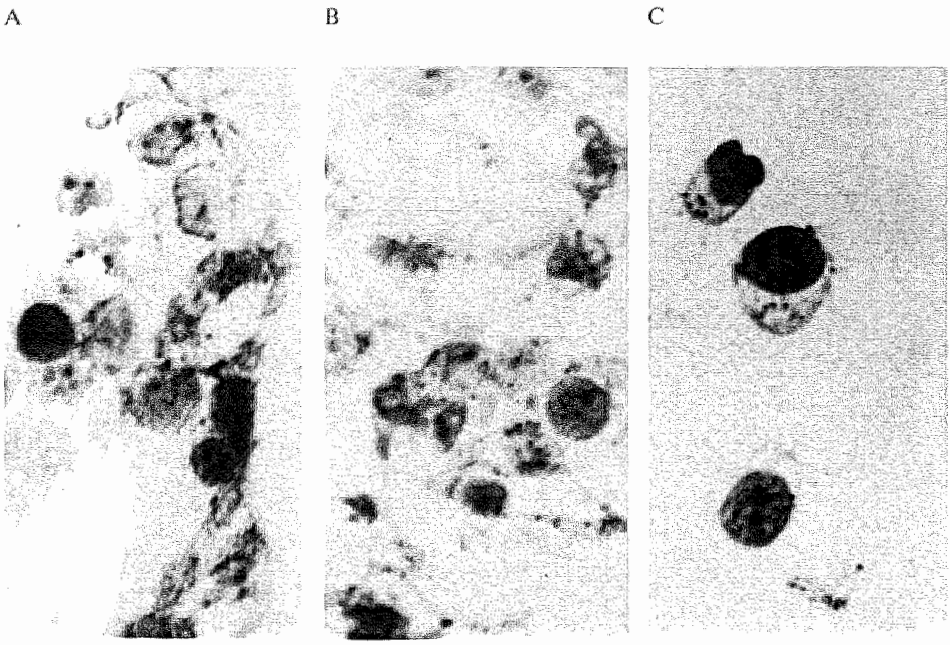

Fig. 5. Frozen sections (A and B) and cytospin preparation (C) of WISH and KB cells (immunoperoxidase 900 x).
A + B. Cross-species reactive polyclonal antiserum to type IV collagen.
C. Monoclonal human specific antibodies to type IV collagen.
Granular intracytoplasmatic staining.

nude mice and rats, using cross-species reactive polyclonal antibodies as well as human specific monoclonal antibodies to type IV collagen. We observed type IV collagen immunoreactivity in BM of the extracellular matrix of a primary colonic carcinoma and of the xenografted cell-line (5583-S) using cross-species reactive polyclonal anti-type IV collagen antiserum. However, with human specific monoclonal anti-type IV collagen antibodies immunoreactivity was only detected in the primary colonic carcinoma but not in the xenograft. In xenografted normal human colonic mucosal fragments the BM of epithelium, endothelium and smooth muscle did react with polyclonal as well as monoclonal antibodies. Neither by immunoblotting of the supernatant nor by immunohistochemistry could we detect in vitro production of type IV collagen by 5583-S cells. In vitro production of type IV collagen, however, was found in WISH and KB cells by these methods. In xenografts of WISH and KB cells polyclonal as well as monoclonal antibodies did stain BM like structures, vascular BM showed only immunoreactivity with polyclonal antibodies. In xenografts of 5583-S cells BM therefore lack a human type IV collagen epitope, which can be detected in primary cancer tissues as well as in the human colonic mucosa xenografts, which included stromal elements. Tumor cells which do produce type IV collagen

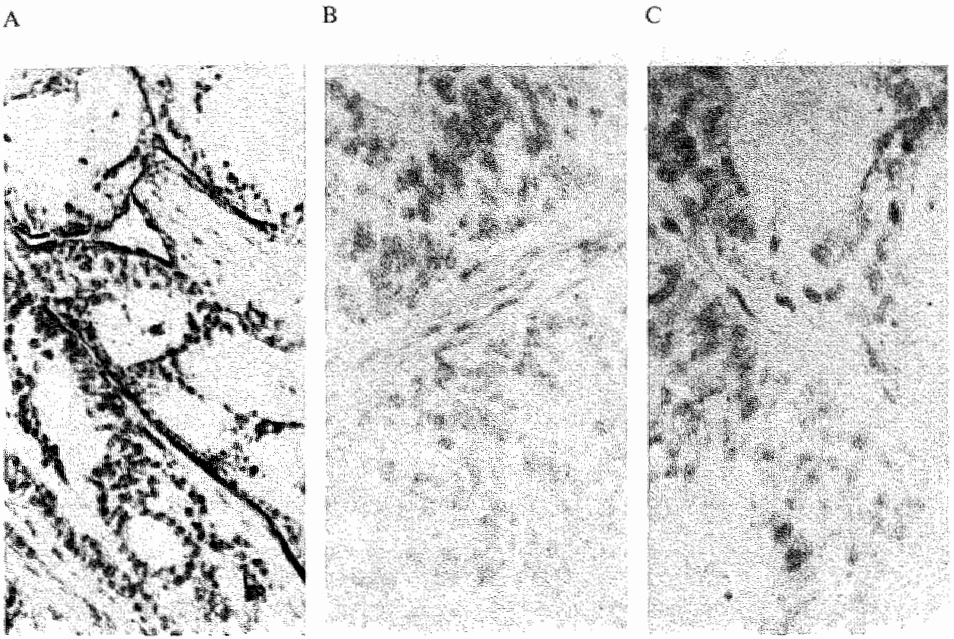

Fig. 6. Frozen sections of xenografted tumor derived from tumor cell-line 5583 (immunoperoxidase 340x).
A. cross species reactive polyclonal antiserum to type IV collagen
B. monoclonal human specific antibody to type IV collagen (1042)
C. monoclonal human specific antibody to type IV collagen (1043)
The human specific monoclonal antibodies to type IV collagen do not react with type IV collagen in the tumor.

in vitro, however, also deposit human type IV collagen in a xenograft. The most obvious explanation for these findings would be that in tumors which lack the ability to produce BM components in vitro, but do show deposition of BM in vivo, this element of the extracellular matrix is derived from the mesenchymal stroma. Two alternative explanations should be considered. Firstly, neoplastic cells might produce an abnormal type IV collagen molecule, which lack the human specific epitope. The reactivity of the primary tumors with the monoclonal antibodies argues against this possibility. Secondly, under transplantation conditions the expression of the human specific epitope might be lost. The persistence of the reactivity in the normal colonic mucosa xenografts, however, rules out this explanation. It is conceivable that in cancer tissues in the in vivo situation mesenchymal stroma regulates the production of BM components by neoplastic cells. Coculturing experiments of human carcinoma cells with murine and human fibroblasts might further clarify whether or not mesenchymal stroma is involved in the regulation of the production of BM constituents and their assembly to a structurally recognizable BM in vitro.

A B

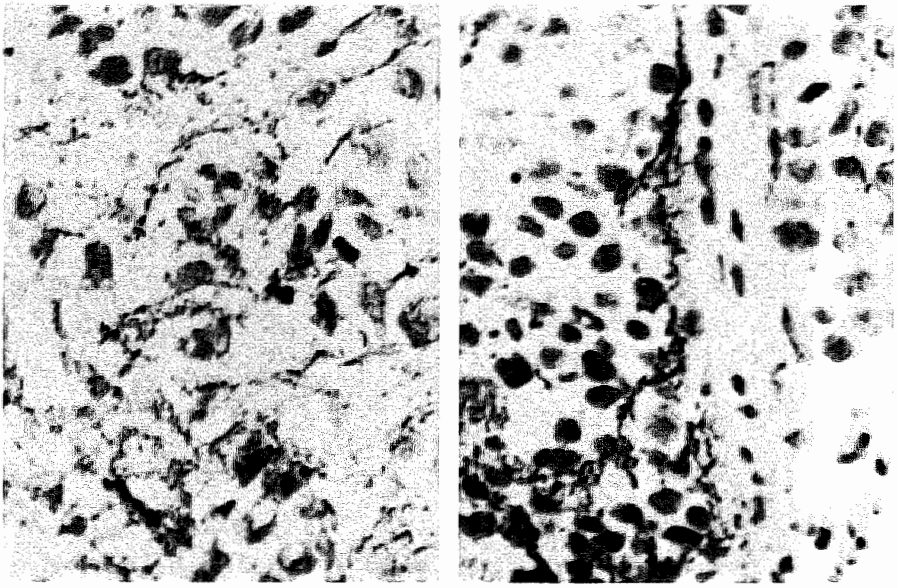

Fig. 7. Frozen sections of xenografts of WISH and KB cells in nude rats (immunoperoxidase 560x).
A. Monoclonal human specific antibody (1042) to type IV collagen applied to WISH xenograft.
B. Monoclonal human specific antibody (1043) to type IV collagen applied to KB xenograft.
BM deposition at the tumor stromal border as well as intercellular.

Our preliminary results with WISH and KB cells grown in collagen lattices, however, do not support this contention. In summary, the experiments described in this report indicate that in colonic carcinoma cells 5583-S BM in the extracellular matrix may be exclusively of stromal origin. Additional experiments are required to elucidate the mechanisms of BM deposition in epithelial neoplasms.

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CHAPTER 4

Type IV collagen immunoreactivity in colorectal cancer: Prognostic value of basement membrane deposition *

4.1 INTRODUCTION

Invasion and metastasis are hallmarks of malignant neoplasia and frequently determine the course of the disease. Invasion precedes metastasis and interactions between tumor cells and the extracellular matrix are involved in this dynamic process (1-3). In carcinomas the first step is penetration through the epithelial basement membrane (BM). BM degrading proteases such as type IV collagenase play an important role in this process (4,5). BM, however, are not static structures, that can only be destructed, but may also be deposited in tumor tissue. Conceivably the presence of BM in a neoplasm might be an expression of the type of interaction of tumor cells with the extracellular matrix. Such information might be related to the biological behavior of the neoplasm.
In this context Stenbäck et al. studied BM in endometrial adenocarcinomas. Well differentiated tumors contained extensive and continuous BM, whereas poorly differentiated adenocarcinomas were characterized by the absence of continuous BM (6). Very few reports have been published concerning BM in colorectal cancer. Burtin et al. showed by immunofluorescence, using antibodies to the BM components type IV collagen and laminin, an almost complete absence of BM at the periphery of colorectal adenocarcinomas (7). Lymph node metastases mostly showed less extensive BM deposition than the primary tumor (8). We have previously found that colonic adenomas with severe dysplasia show irregular defects in their epithelial BM (3). Forster studied the relationship between the deposition of BM and metastatic propensity in colorectal carcinomas by immunohistochemical staining for laminin (9). Limited deposition of BM in primary tumors correlated significantly with a higher incidence of distant metastases and reduced patient survival (10). We have performed a similar study, using immunohistochemistry to detect type IV collagen in BM in colorectal carcinomas. Patterns of BM deposition were correlated with macroscopical (Dukes' stage) and microscopical (degree of differentiation) information and with follow up data.

4.2 MATERIAL AND METHODS

For this study we used clinicopathological information and tissuespecimens of 350 cases of colorectal carcinoma, collectedprospectively in a multicenter study (11).

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Accepted for publication in Cancer

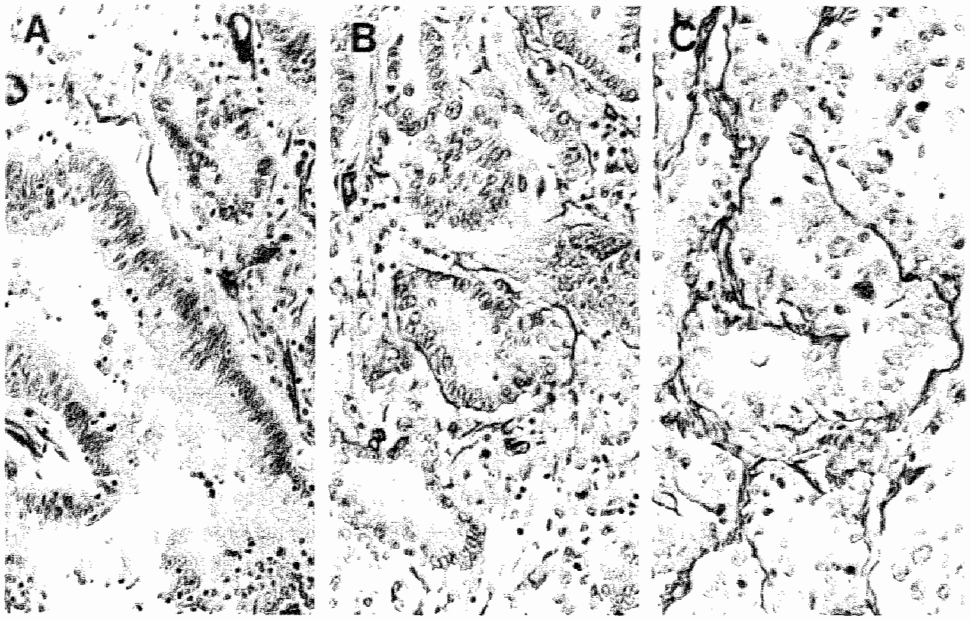

Fig 1. Variation in BM deposition.
A; Limited BM deposition. B; Moderate BM deposition. C; Extensive BM deposition.

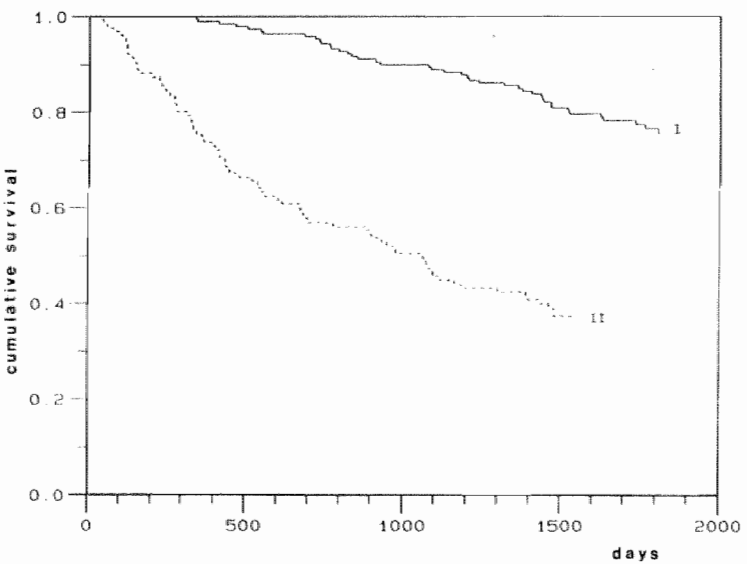

Fig 2. Survival data of Dukes' stages A/B versus stages C/D.
I; Dukes' stages A and B. II; Dukes' stages C and D (log rank test $p<0.01$).

4.2.1 Case material

Staging of the tumors was performed according to the Dukes' classification as modified by Turnbull (12). Stage A tumors were confined to the bowel wall; stage B tumors extended into the pericolic fat; stage C tumors had regional lymph node metastasis and stage D tumors either invaded into adjacent organs or had distant metastases. All clinicopathological data were stored in a computerized database. At regular intervals follow-up data, including survival information, were collected.

4.2.2 Immunohistochemistry

For histological and immunohistochemical evaluation cross sections trough the center of the tumor, at the site of the deepest invasion, were selected. Routinely formalin fixed and paraffin embedded tissue blocks were sectioned at 4 μm. Paraffin sections were deparaffinized, rehydrated and pretreated with pepsin (0.1 % in 0.1 M HCl, for 30 min at room temperature) to restore immunoreactivity to type IV collagen (13). After blocking of endogenous peroxidase (20 min. in methanol with 0.3 % H_2O_2) and washing in PBS (3x5 min.) the sections were incubated with anti type IV collagen antiserum (diluted 1:200 in PBS with 1% BSA) for 1 hour at room temperature in a moist chamber. After washing in PBS the sections were incubated with peroxidase labeled goat anti rabbit Ig antibodies (1h RT). After final washing with PBS, a diaminobenzidine-H_2O_2 substrate was used to visualize the immunoreactivity.

As primary antibody a cross-species reactive anti-type IV collagen antiserum raised in rabbits was used. Immunoreactivity and specificity of this antiserum has been described elsewhere (13). Briefly, under immunohistochemical conditions the antibodies reacted only with type IV collagen and not with types I,III and V collagen nor with laminin. All BM in normal tissues could be visualized by immunostaining with this antibody.

The amount of immunoreactivity at the tumor-stromal interface was scored semiquantitatively in the tumor center only, as in the tumor periphery invariably limited BM was found. When more than 75% of the linear tumor cell-stromal interface showed type IV collagen immunoreactivity this was scored as extensive BM deposition. When this amounted between 25% and 75% the score was moderate and less than 25% was classified as limited BM deposition. These patterns are illustrated in Fig 1.

In all cases the overall extent of BM deposition at the tumor cell-stroma interface of the whole section, covering a large tumor area at the site of deepest invasive growth, was scored.

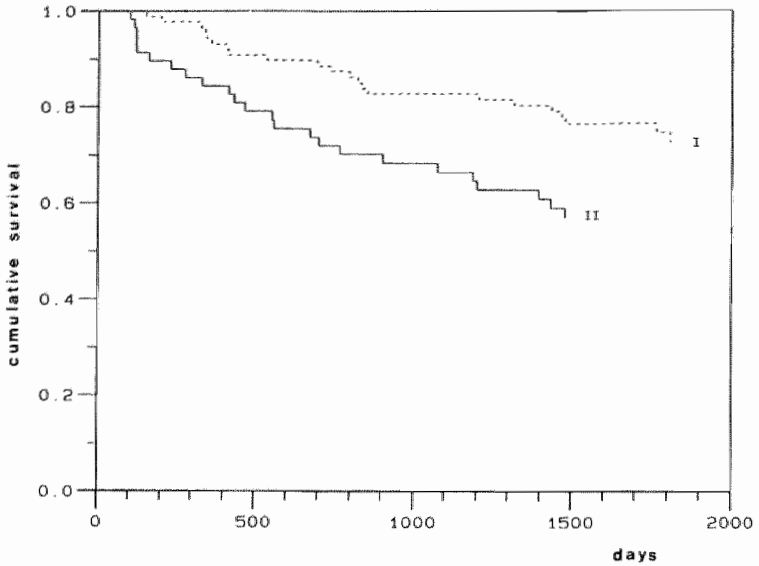

Fig 3. Survival data of extensive and moderate versus limited BM deposition.
I; Extensive and moderate BM deposition. II; Limited BM deposition (log rank test $p<0.01$).

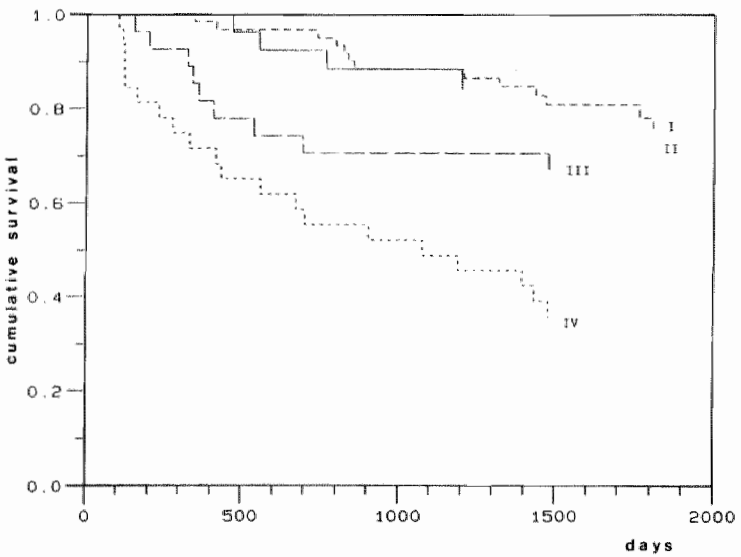

Fig 4. Survival data after stratification of Dukes' stages and BM deposition.
I; Dukes' stages A and B with extensive and moderate BM deposition. II; Dukes, stages A and B with limited BM deposition. III; Dukes' stages C and D with extensive and moderate BM deposition. Note the occurence of a plateau phase after two years. IV; Dukes' stages C and D with limited BM deposition (log rank test $p<0.01$).

4.2.3 Statistical analysis

Correlations between parameters in cross-tabulations were analyzed by the Chi-square test. Survival data were analyzed by a logrank test (14). Death due to postoperative complications, including all deaths within thirty days after operation, and non-disease related deaths were excluded for analysis of survival data.

4.3 RESULTS

4.3.1 Immunohistochemistry

Paraffin sections of all 350 colorectal carcinomas were stained. Subendothelial BM, and BM surrounding individual smooth muscle fat and Schwann cells, were regarded as internal control in the tumor sections. Only when these BM stained properly, the immunostaining was considered to be appropriate. In only 163 cases reliable immunohistochemistry could be performed, due to our inability to restore type IV immunoreactivity by pepsin pretreatment. Cases with unreliable immunohistochemistry were found to be randomly distributed over all Dukes stages.

4.3.2 Clinicopathological data

In the periphery of all colorectal carcinomas, invariably a limited BM deposition was found. However, the central parts of the tumors showed a markedly variable immunoreactivity at the tumor/stroma interface, varying from limited to extensive BM deposition (Fig 1). For statistical reasons extensive and moderate BM deposition were combined and compared with limited BM deposition in a cross-table with Dukes' stages A and B (no metastasis), and Dukes' stage C and D (metastasis). The results are shown in Table 1. In Dukes', stages A and B the cases with extensive and moderate BM deposition appeared to be overrepresented and those with limited BM deposition were underrepresented. In Dukes' stages C and D the opposite pattern occurred ($p < 0.01$).
Overall survival data of all 350 patients classified into Dukes' stages A and B and Dukes' stages C and D cases were analyzed. Actuarial survival curves are shown in Fig 2. Patients with Dukes' stage C or D carcinomas appeared to survive significantly shorter ($p < 0.01$) than those with Dukes'stages A and B carcinomas. Survival data of cases with extensive and moderate BM deposition were compared to those with limited BM deposition (Fig 3). Patients with limited BM deposition in colorectal carcinomas showed a significantly shorter survival ($p < 0.01$), compared to those with moderate and extensive BM deposition. Stratification of the cases for limited versus moderate/extensive BM deposition and Dukes' stages A/B and C/D resulted in the survival curves shown in Fig 4. In Dukes' stages A and B, BM deposition did not correlate with survival. However, in Dukes' stages C and D survival was signifi-

Table 1. Cross-table; Dukes' stages versus BM deposition.

			BM deposition		
			E/M	LIM	
Dukes' stages A/B	number		67	30	97 number
	row	%	69.1	30.9	59.5%
	column	%	67.7	46.9	
	total	%	47.1	18.4	
Dukes' stages C/D	number		32	34	66 number
	row	%	48.5	51.5	40.5%
	column	%	32.3	53.1	
	total	%	19.6	20.9	
Column total	number		99	64	163 number
		%	60.7	39.3	100%

cantly shorter ($p<0.01$) in cases with limited BM deposition. In this group cases with Dukes' stages C and D were equally represented (7 Dukes' C versus 8 Dukes' D), which excludes the possibility of overrepresentation of Dukes' D cases. Survival in the group with moderate and extensive BM deposition reached a plateau phase after two years (fig 4).

4.4 DISCUSSION

In carcinomas, a dynamic interaction occurs at the interface between tumor cells and the surrounding mesenchymal stroma. Collagenases, including specific type IV collagenase, and other proteases such as plasminogen activators, cathepsins, and heparanases form a cascade system of enzymes facilitating extracellular matrix breakdown (4,15,16). Conversely, as a host reaction to the invading neoplasm extracellular matrix components, including BM material and interstitial collagens may, also be deposited around the tumor cells (17,18). Stromal desmoplastic reaction around tumor cells, in which myofibroblasts are involved, might play a role in BM deposition at the tumor-stromal interface. It is tempting to speculate, on the basis of the previously reported information and our own studies, that extensive BM deposition in a neoplasm is a sign of competent host response or of limited invasive potential and might indicate low metastatic capability.

Against this background we studied BM deposition by immunohistochemical demonstration of type IV collagen in colorectal carcinomas. Consistent with the hypothesis formulated above, in the invasive periphery of the tumor BM deposition was scarce or completely absent. In the tumor center, however, a highly variable pattern of BM deposition occurred. Why some tumors do and others do not show

BM deposition is currently unknown. It is likely that limited BM deposition is caused by highly active protease secretion by tumor cells together with low type IV collagen synthesis or inability of the tumor cells to deposit type IV collagen into an organized BM.

The most interesting finding was the correlation of BM deposition pattern with prognosis. In Dukes' stages A and B cases moderate and extensive BM deposition occurred more frequently, but within these prognostically favorable stages, the amount of BM deposition had no influence on patient survival. In contrast, in Dukes' stages C and D, limited BM deposition occurred more frequently. Cases with limited BM deposition showed a highly significant shorter survival ($p < 0.01$). Cases with extensive and moderate BM deposition showed a plateau phase in the survival curve after two years of follow up. Death during the first two years could be explained by cases in Dukes' stage D and by micrometastases in the liver, already present at the time of surgical resection of Dukes' stage C cases. Cases with limited BM deposition showed a continuously downward course of the survival curve, which could be explaned by locoregional recurrences causing liver metastasis after a longer postoperative interval. These findings suggest that immunostaining of BM in Dukes' stage C differentiates tumors with relatively low invasive and metastatic capacity from tumors with a high invasive and metastatic capacity.

Poorly differentiated tumors showed a trend towards limited BM deposition and well differentiated tumors towards moderate/extensive BM deposition. These findings are in agreement with other studies (6,7,9,10). Because of the limited number of well or poorly differentiated carcinomas stratification according to tumor grade could not be performed.

Absence of the adhesive glycoprotein laminin, which occurs only in the BM, has been postulated as an explanation for the higher incidence of metastases and subsequent lower patient survival in colorectal carcinomas (10). However, our finding that type IV collagen deposition shows a similar correlation with survival, suggests that BM in carcinomas may also have a barrier function. In conclusion, we have shown that the pattern of BM deposition in the center of colorectal carcinomas is highly variable. Deposition of appreciable amounts of BM material appears to be a prognostically favorable sign. How patterns of BM deposition can influence clinical behavior is an interesting subject for further studies.

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CHAPTER 5

Basement membranes in bronchogenic squamous cell carcinoma: An immunohistochemical and ultrastructural study *

5.1 INTRODUCTION

Invasive growth of cancer cells is a complicated process in which specific interactions between tumor cells and the extracellular matrix are involved (1). In tumor pathology, penetration of the basement membrane (BM) by carcinoma cells, followed by migration into the interstitial stroma, is often regarded as the first sign of invasive growth (2-4). Consequently, at the point of invasion the BM is usually discontinuous (5, 6). In solid organs tumor cells invade into the surrounding mesenchymal stroma. In the lung, however, specific anatomic characteristics might influence the pattern of invasion of neoplastic cells. Axial connective tissue surrounds the bronchi and large vessels, whereas peripheral connective tissue underlies the pleural mesothelium and extends into fissures. The delicate connective tissue in the alveolar walls forms the bridge between the axial and peripheral connective tissue (7). In the initial stage of bronchogenic squamous cell carcinomas, invasive growth in the lung is comparable to invasion in solid organs. However, when carcinoma cells gain entrance into the intra-alveolar compartment, the functional barrier formed by the interstitial stroma, alveolar BM, and epithelium is lost and tumor cells actually grow in the extracorporal space. This could lead to striking differences of invasive growth patterns between carcinomas in solid organs and primary as well as metastatic lung tumors. Recently, these differences have been described at the ultrastructural level in bronchogenic squamous cell carcinomas (8, 9).
Hitherto, at the light microscopic level, no reports on the role of BM in invasive growth in the lung are known. Visualization of the BM at the light microscopic level has been attempted with conventional histochemical methods such as periodic acid Schiff staining (PAS) and silver impregnation techniques like Gomori's reticulin staining. These staining methods are not specific for BM, because structural proteins in the interstitial stroma are also stained (10, 11). Since antibodies to BM constituents have become available, immunolocalization of BM at the light, as well as the electron microscopic level is possible (12-15).
In the present report we studied BM patterns in squamous cell carcinomas of the lung by immunohistochemistry, using antibodies to type IV collagen and laminin, and by electron microscopy. The following questions were addressed: 1.are the results of immunohistochemistry of BM components in bronchogenic squamous cell

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Submitted to Ultrastructural Pathology.

Fig 1 Western blot of mouse laminin, reacted with polyclonal rabbit anti mouse laminin antibodies.

carcinomas and electron microscopy comparable; 2.does invasive growth into alveolar septa in the periphery of squamous cell carcinomas of the lung occur, and if so, is it detectable at the light microscopic level; 3.which cells are present in the stromal compartment in the tumor center and what is the relationship of the tumor cells to the stromal compartment regarding BM deposition; 4.does inappropriate intercellular BM deposition occur, and is intracellular immunoreactivity to BM components detectable, in squamous cell carcinoma of the lung.

5.2 MATERIALS AND METHODS

5.2.1 Tissues

Tissue specimens of 27 squamous cell carcinomas of the lung were collected from lobectomy and pneumonectomy specimens, which were freshly obtained from the operating theatre. Unselected cases were used, the only criterion for inclusion in the study being the presence of cell bridges and/or keratinization, which are the light microscopic criteria for squamous cell carcinomas as given by the WHO (1981). Of each tumor, tissue was frozen in isopentane quenched in liquid N2 and stored at -70°C. Specimens from the peripheral and central parts were fixed in Karnovsky's fixative for electron microscopy, the remaining tissue was fixed in 4% neutral

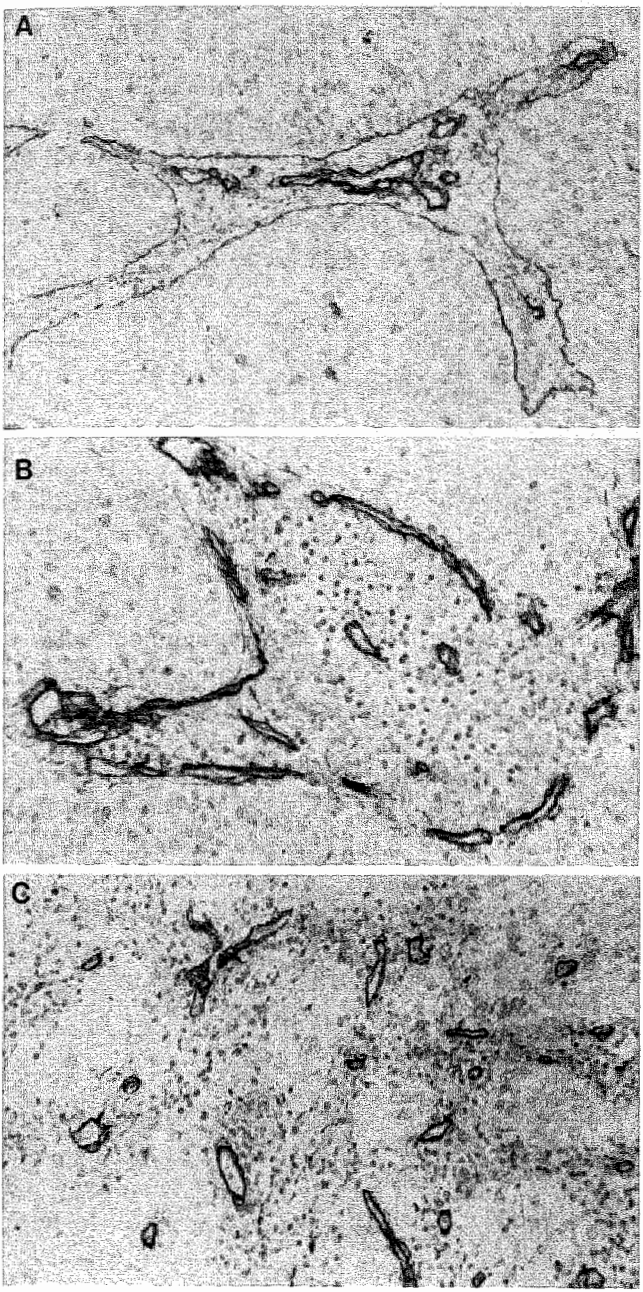


Fig 2 Frozen sections of bronchogenic squamous cell carcinoma reacted with polyclonal antibodies to type IV collagen (immunoperoxidase 240x).
A. Extensive BM deposition.
B. Moderate BM deposition.
C. Limited BM deposition.

buffered formaldehyde. After fixation in Karnovsky's fixative, for at least one hour the specimens were divided into 3x3x3 mm blocks (i.e. larger than usual for electron microscopy), postfixed with OsO_4, rapidly dehydrated using 2,2-dimethoxypropane (16), and embedded in LX 112 resin. For immuno-electron microscopic demonstration of type IV collagen, samples from some of the tumors were fixed in 2% paraformaldehyde and embedded in Lowicryl K4M at -35°C. The remaining tissue was sectioned for histopathological examination, after routine fixation in formalin and embedding in paraffin. One paraffin block of each case, containing peripheral as well as central parts of the tumor, was selected.

5.2.2 Antibodies

Polyclonal antibodies to human type IV collagen raised in rabbits, were applied to frozen and paraffin sections. Type IV collagen, used for immunization, was isolated from human placenta. Immunoreactivity and immunospecificity was tested by ELISA and immunoblotting. The polyclonal antiserum showed in ELISA a very weak immunoreactivity to type V collagen. However, by immunohistochemistry no immunoreactivity to interstitial stroma could be detected. The polyclonal antibodies stained all BM in a variety of normal human tissues. To obtain immunoreactivity, paraffin sections required enzyme preincubation. The best results were obtained with pepsin digestion. The details of these procedures have been described elsewhere (17).
Antibodies to laminin were raised in rabbits, immunized with commercially available mouse laminin (E/Y Laboratories) isolated from EHS (Englebreth-Holm-Swarm) mouse tumor (18). In antigen spot tests these antibodies lacked any immunoreactivity to type I,III,IV and V collagen. Western blotting studies revealed immunoreactivity at 200 Kd as shown in Fig 1. To obtain immunoreactivity, paraffin sections required enzyme preincubation as for antibodies to type IV collagen.

5.2.3 Immunohistochemistry

Frozen sections were fixed 20 min in acetone (-20°C) and endogenous peroxidase was blocked in 0.3% H_2O_2 in methanol, 20 min, RT. After washing with PBS (3x5min) antibodies to BM components (diluted in PBS 1% BSA) were incubated for 1 h in a moist chamber. After washing with PBS the sections were incubated with peroxidase labeled rabbit anti mouse antibodies, for 1h at RT. After final washing with PBS, a diaminobenzidine-H_2O_2 substrate was used to visualize the immunoreactivity.
After deparaffinization, rehydration and enzyme preincubation, paraffin sections were treated like frozen sections, excepting fixation in cold acetone.
For immuno-electron microscopy thin sections of Lowicryl embedded tissues were

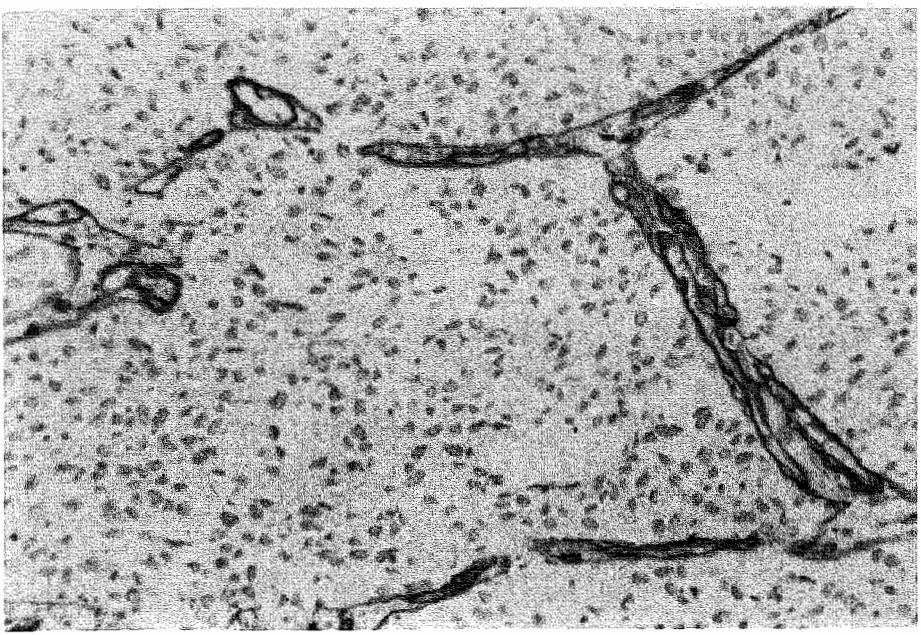


Fig 3 Frozen section of bronchogenic squamous cell carcinoma reacted with polyclonal antibodies to type IV collagen (immunoperoxidase 240x). Alveolar pattern in the periphery of the tumor.

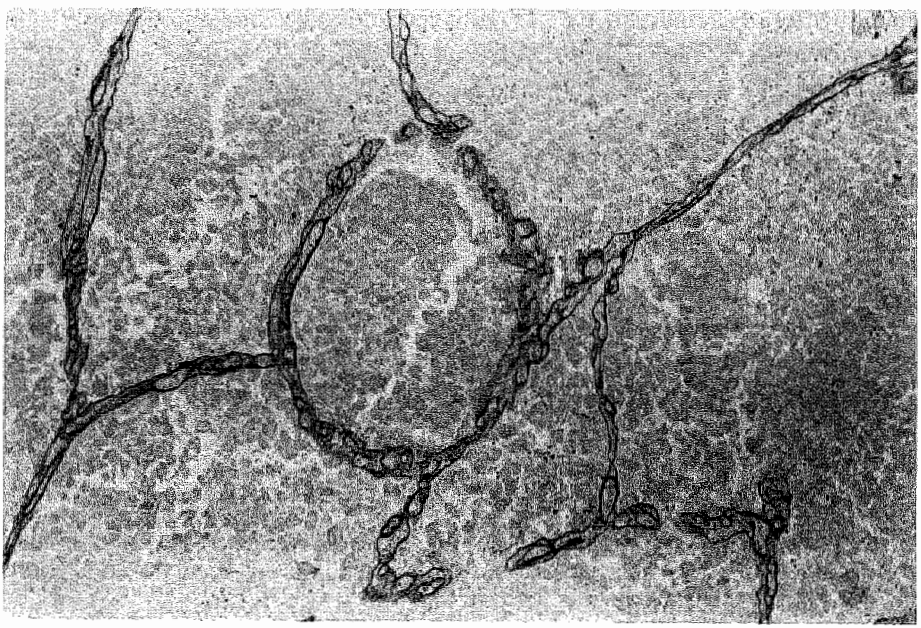


Fig 4 Paraffin section of bronchogenic squamous cell carcinoma reacted with polyclonal antibodies to type IV collagen (immunoperoxidase 60x).

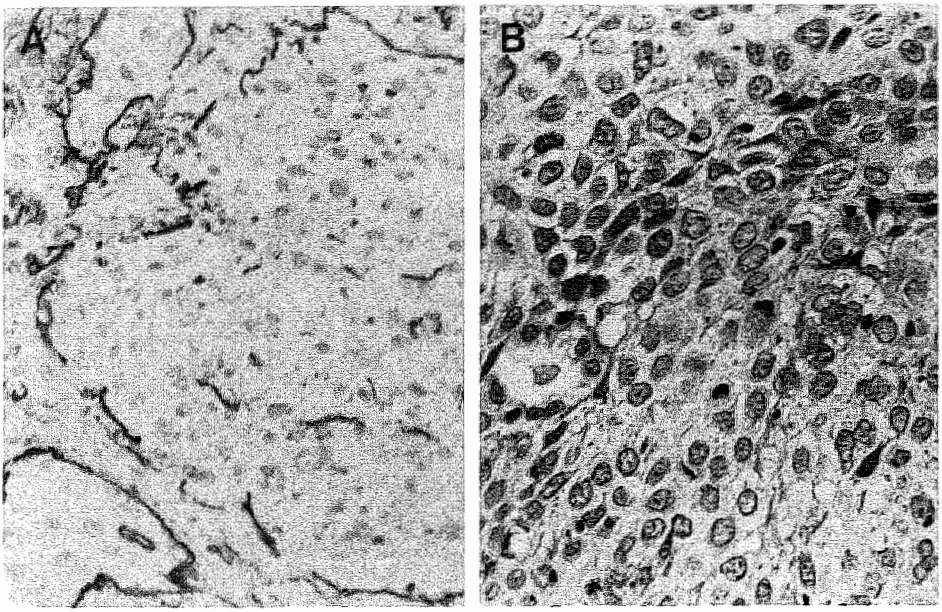


Fig 5 Frozen section (A) and paraffin section (B) of bronchogenic squamous carcinoma reacted with polyclonal antibodies to type IV collagen (immunoperoxidase, A 240x, B 375x). A, intercellular BM deposition. B, granular immunoreactivity.

incubated with polyclonal anti type IV collagen antibodies and subsequently with goat anti rabbit IgG, labelled with 15 nm gold particles (Janssen).
BM staining at the tumor-stroma interface was scored semiquantitatively. More than 75% immunoreactivity at the tumor-stromal border was scored as extensive BM, between 25 and 75% as moderate, and lower than 25% as limited BM expression (Fig 2).

5.3 RESULTS

In normal lung tissue all BM were stained with antibodies to type IV collagen and laminin in an identical pattern. Antibodies to type IV collagen gave a slightly higher staining intensity than antibodies to laminin.
In all tumors studied, the peripheral tumor tissue expanded into the alveolar spaces, while in close contact with the BM of the preexisting septa (Fig 3). Despite this close contact, the BM lacked discontinuities, and the tumor cell groups were surrounded by a continuous BM. Whereas invasion of the septa was never detected, tumor cells located in alveolar pores were occasionally observed, the adjacent alveolar walls showing uninterrupted BM (Fig 3). Even in necrotic areas, the alveolar pattern was clearly recognizable (Fig 4). In a few of the squamous cell carcinomas originating in large, centrally located bronchi, invasive growth was restricted to the peribronchi-

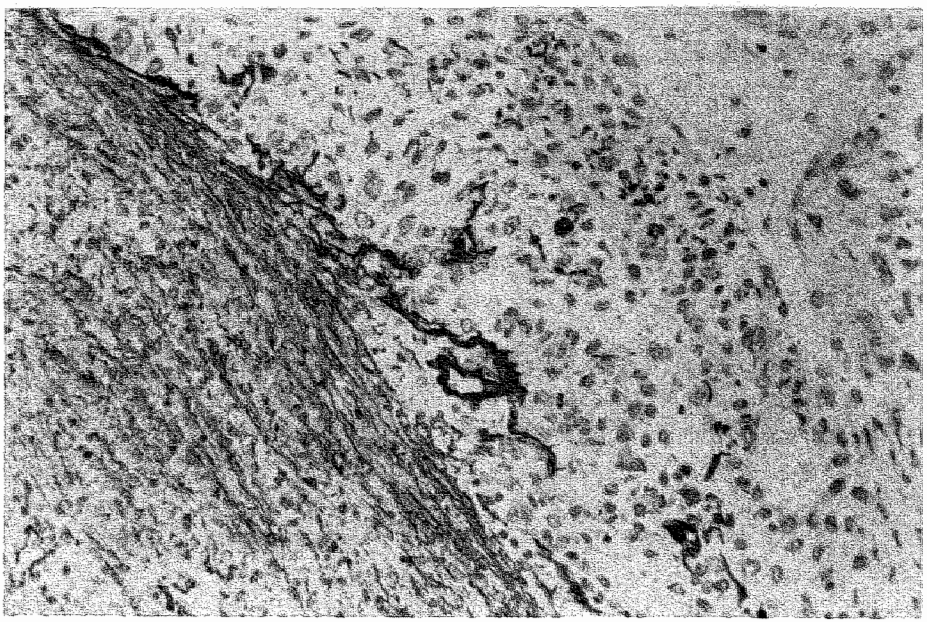


Fig 6 Frozen sections of bronchogenic squamous cell carcinoma reacted with polyclonal antibodies to type IV collagen (immunoperoxidase 240x). Irregular pericellular immunoreactivity in the stroma adjacent to the tumor.

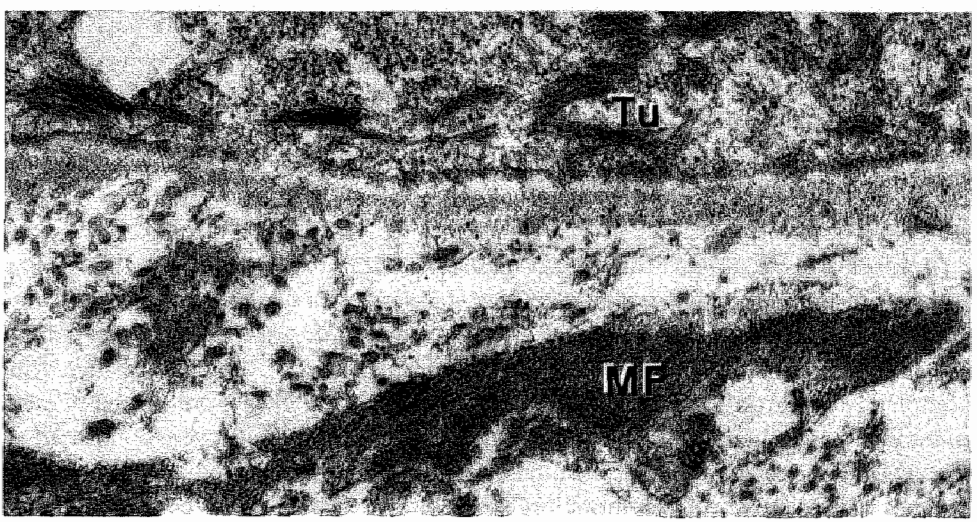


Fig 7. Immuno-electron microscopic demonstration of type IV collagen. Label is seen over lamina densa of BM separating tumor cells (Tu), characterized by tonofibrils, from stroma. Some granules are also found near surface of myofibroblasts (MF), characterized by microfilaments (36000x).

al stroma. Consequently, these cases lacked a peripheral alveolar pattern.
In contrast to the tumor periphery, the more centrally located tumor regions consisted of irregular groups and strands of tumor cells separated by abundant stroma. In these regions, there was a highly variable BM deposition at the tumor stroma interface, ranging from continuous in about 50% of the tumors to almost completely absent in others (Fig 2). Small tumor cell clusters apparently invading the stroma showed the same variability as the larger tumor cell groups. There was no correlation between the grade of differentiation and the extent of BM deposition around the deeper tumor cell groups (e.g. some poorly differentiated tumors expressed almost continuous BM).
In the peripheral as well as the central regions of many tumors, some BM immunoreactivity was also found within the tumor cell groups. Occasionally, this reactivity was apparently due to the presence of BM associated with preexistent alveolar structures that were trapped by the tumor cells. In other instances, the immunoreactivity seemed to be located inside the tumor cells (Fig 5). In many instances, however, the exact position of this BM immunoreactivity could not be determined light microscopically.
The stroma present in the deeper tumor areas consisted of loose connective tissue containing blood vessels, spindle cells and inflammatory infiltrate. The vessels had a distinct, continuous BM, whereas the spindle cells showed irregular pericellular immunoreactivity (Fig 6).
The ultrastructural observations were largely in agreement with the light microscopic results. Immuno-electron microscopy using antibodies to type IV collagen confirmed that the lamina densa observed electron microscopically corresponded to the immunoreactive structures identified by light microscopy (Fig 7).
Only in a few tumors there was a slight disagreement between the extent of BM presence as determined by the two techniques; these differences were almost certainly due to the sampling errors which present a potential danger to any ultrastructural investigation, even when relatively large tissue blocks are used as in the present study.
There was a slight but consistent morphologic difference between the BM in the tumor periphery and that in the tumor center: whereas the BM in the periphery, which obviously represented the preexisting alveolar BM, generally lacked a lamina lucida (8), the BM surrounding the deeper tumor cell groups, which may have been completely or partially deposited by the tumor cells, were always composed of a lamina lucida and a lamina densa. In all instances, the tumor cells were connected to the BM by means of hemidesmosomes. It should be emphasized that even small patches of BM detected in tumors largely lacking a BM showed frequent hemidesmosomes (Fig 8).
The BM immunopositivity within tumor cell groups detected by light microscopy was found to correspond to one of the three following situations: a. seemingly intracellular BM was found to be located in deep invaginations of the tumor cells; not

Fig 8A. Part of tumor cell with irregular surface that is almost completely devoid of BM.

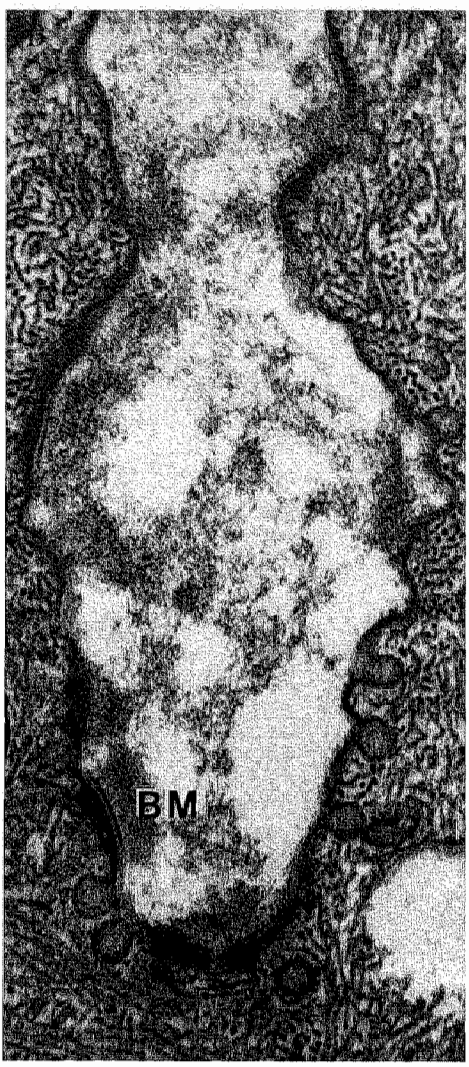


Fig. 8B. Enlargement of deep surface invagination in A. Notice hemidesmosomes (characterized by fine, extracellular, electron-dense line) associated with small patches of BM. (A 18500x, B 73000x).

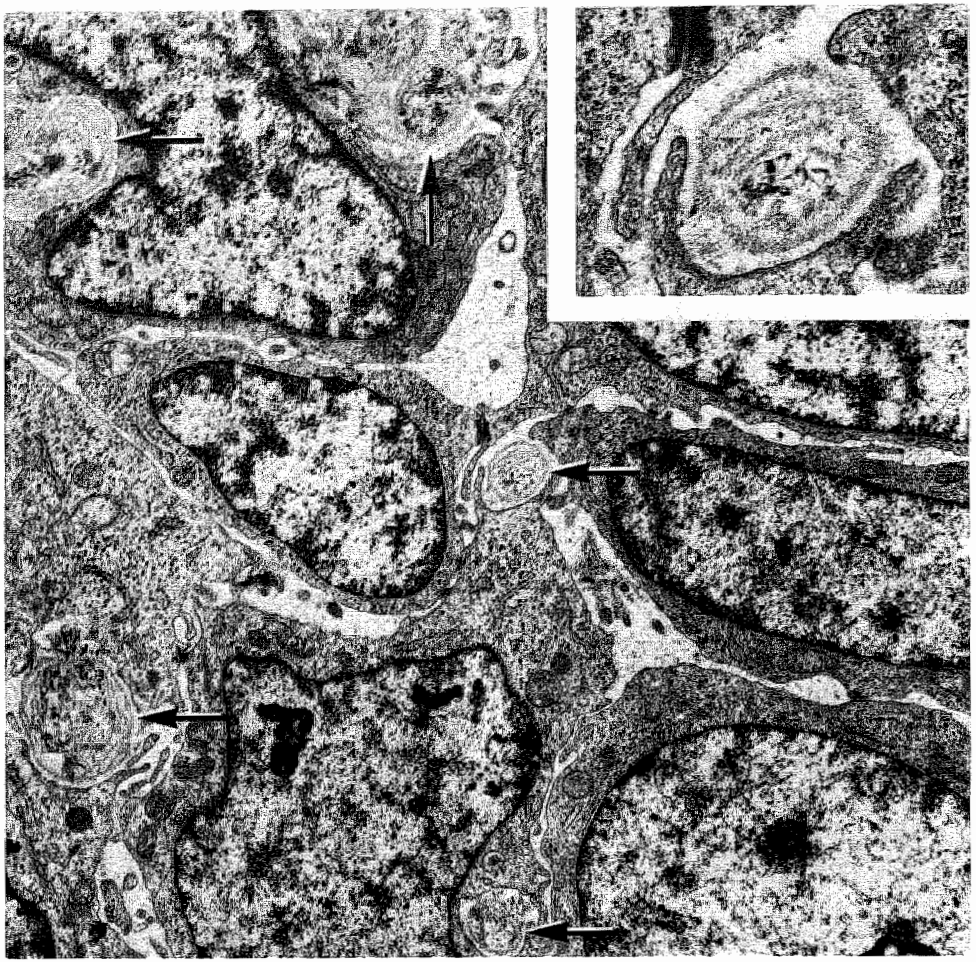


Fig 9. Tumor area with several rounded BM deposits (arrows) between tumor cells. Inset shows enlargement of deposit consisting of several layers of BM around collagen fibers. (7100x, inset 32000x).

infrequently, such small BM deposits were fully surrounded by tumor cell cytoplasm in the plane of the section (Fig 8); b. rounded deposits of BM with associated collagen fibers were found scattered over the tumor cell groups (Fig 9); c. remnants of BM were often attached to individual pneumocytes trapped by the tumor or the aggregates of a few trapped pneumocytes that were too small to be easily recognized by light microscopy (Fig 10).

In one tumor in which the deeper tumor cell groups were virtually devoid of BM,

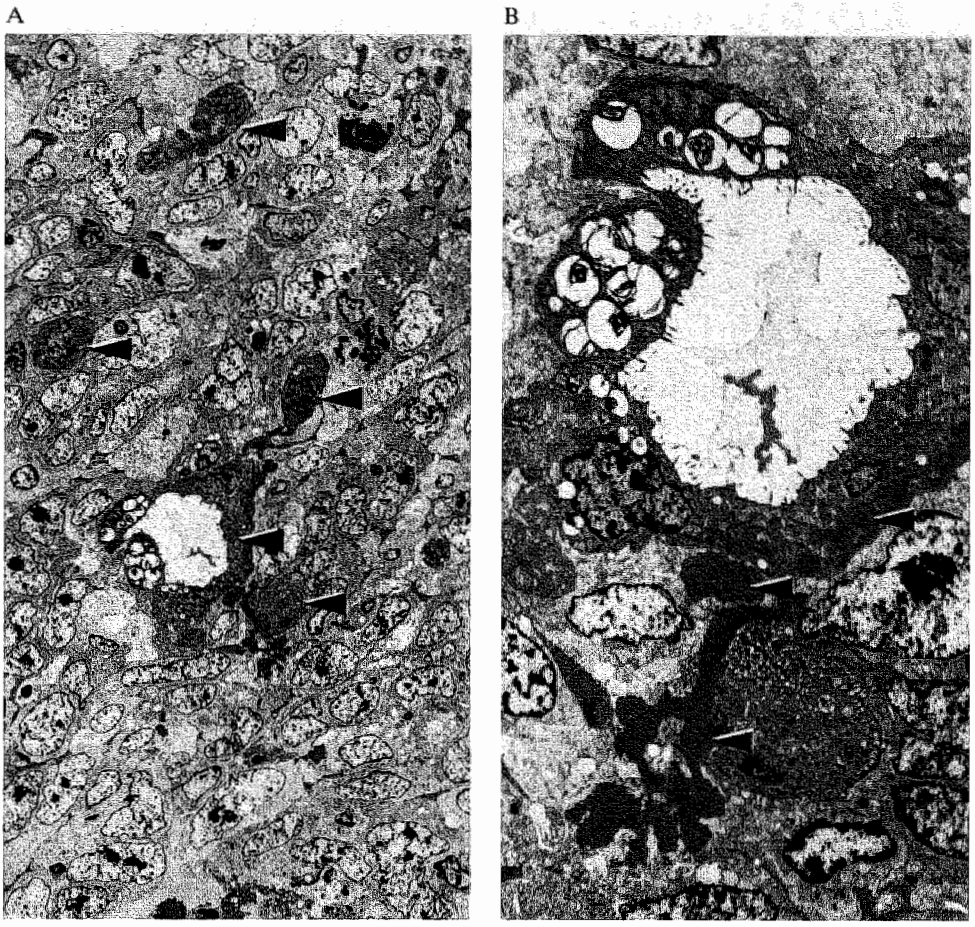
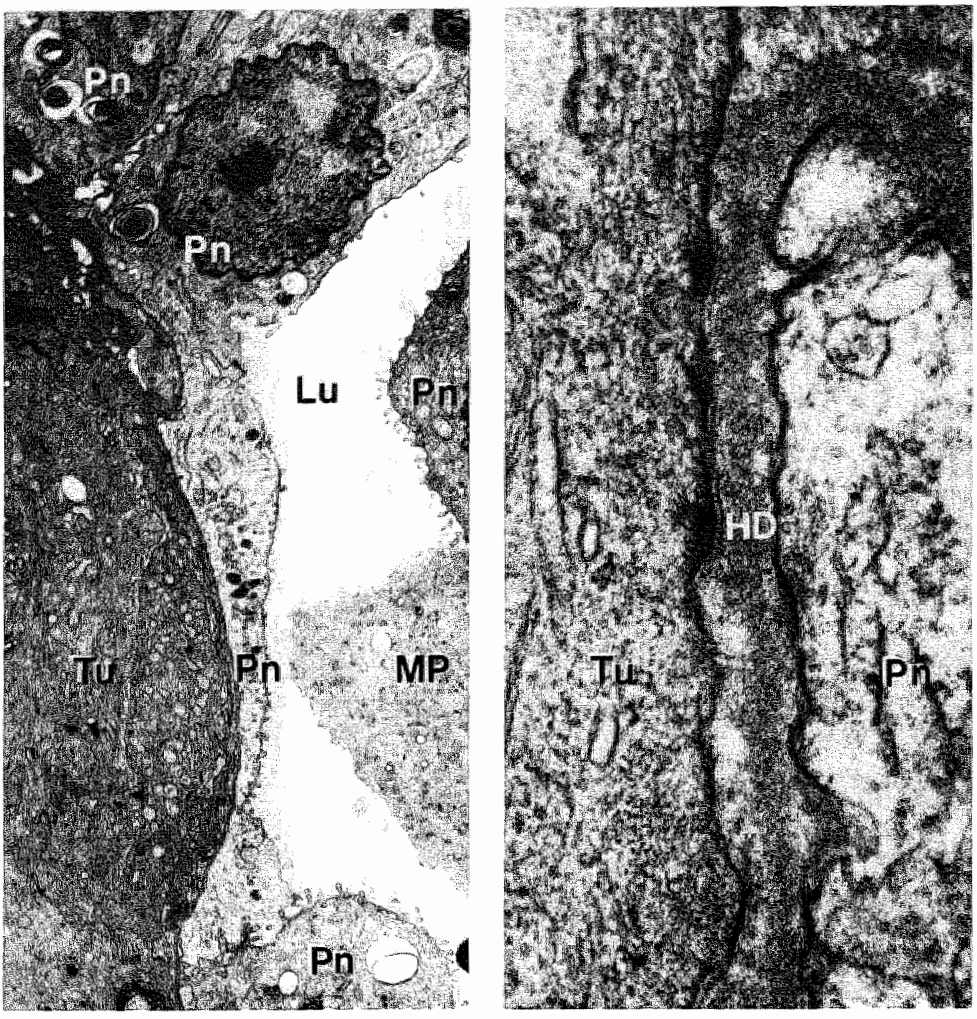


Fig 10A. Survey of field of tumor cells in which individual pneumocytes as well as small groups of pneumocytes with intracytoplasmic lamellar inclusions characteristic of type II pneumocytes and lumen lined with microvilli. Notice conspicuous remnants of BM (arrow heads). (A 850x, B, 2900x).

a search was made for tumor cells in contact with preexisting BM. Examples of tumor cells located at the stromal side of the BM surrounding trapped alveoli were readily found. Interestingly, in this situation the tumor cells exhibited a strong affinity for the BM and formed hemidesmosomes with it, despite the fact that the tumor cells were located at the "wrong" side of the BM (Fig 11).

The spindle cells present in the stroma had all the characteristics of myofibroblasts, i.e., an extensive rough endocytoplasmic reticulum occupying the central cytoplasm and large or small microfilaments arranged along the cell periphery (9). Part of the cell surface was covered by BM-like structure. Other areas, however, were associat-



A *B*

Fig 11. In this particular tumor, surface of tumor cell was virtually devoid of BM, whereas trapped preexistent alveoli had complete BM. A illustrates area where tumor cells (Tu) are situated next to trapped alveolus lined by pneumocytes (Pn). Lu, collapsed alveolar lumen; MP, alveolar macrophage. Detail enlarged in B shows that tumor cells and pneumocytes are separated by continuous BM. Small hemidesmosomes (HD) connect tumor cell to BM. (A 4400x, B, 100000x).

ed with irregular, flocculent deposits or were directly exposed to the surrounding stroma (Fig 12). Immuno-electron microscopy demonstrated that these deposits contained type IV collagen (Fig 7), but the labelling was weaker and far more irregular than that found on epithelial, tumoral, and vascular BM.

5.4 DISCUSSION

The present study corroborates recent ultrastructural findings (8, 9). Both electron microscopy and immunohistochemistry allow reliable evaluation of BM patterns in lung cancer. Immunohistochemical evaluation of BM has the advantage that large areas of the tumors can be studied. In electron microscopy, on the other hand, individual cells and the intercellular relationships can be identified with better detail. The combination of both techniques is a powerful tool in the study of invasive growth. Electron immunohistochemistry may answer questions regarding the decreased synthesis of BM constituents by tumor cells or by adjacent myofibroblasts or other stromal cells. Evaluation of the role of enzymes active in degradation of BM and other extracellular matrix components, using specific antibodies to these enzymes in electron immunohistochemistry, may further help to elucidate the complex events resulting in invasive growth.

Discontinuity of BM is a characteristic of the tumor-cell stroma interface in most carcinomas (3, 4). Tumor cells gain access to adjacent stroma through the BM, either by means of BM degradation with the help of specific enzymes (e.g. type IV collagenase), or by defective deposition of BM at the tumor-stromal border. If BM degradation exceeds BM deposition, interruptions occur. Strikingly, however, in some invasive carcinomas almost continuous BM have been observed (19, 20).

In primary and metastatic carcinomas of the lung, the adjacent alveolar parenchyma is invaded by tumor cells. Migration and growth of tumor cells into the intra-alveolar compartment is not comparable to invasive growth in solid organs because of the absence of connective tissue. Tumor cells were found to interact with the alveolar tissue elements in a remarkably organized way (8): ultrastructurally, junctional complexes occurred between intra-alveolar tumor cells and neighboring pneumocytes. Furthermore, tumor cells were connected to the alveolar BM by hemidesmosomes. Only small protrusions of tumor cells through the alveolar BM could be detected, but frankly invasive growth into alveolar septa was absent. Immunohistochemistry confirmed the persistence of an alveolar tissue architecture in the tumor periphery, characterized by continuous alveolar BM, and absence of invasive growth into alveolar septa. Our immunohistochemical and ultrastructural findings therefore imply that at the tumor periphery expansive intra-alveolar tumor growth occurs, without overt signs of invasive growth and consequently retention of preexisting alveolar structures. For their metabolic needs tumor cells depend on diffusion of nutrients and oxygen from the vessels in the alveolar wall. Consequently the tumor cores in the alveoli often were necrotic, presumably due to nutritional depri-

Fig 12. Stromal myofibroblasts with irregular BM-like material along surface (26000x).

vation. In the center of the tumors a totally different pattern was found. Usually an extensive desmoplastic reaction was present without remnants of the preexisting alveolar tissue pattern and furthermore, BM staining patterns were highly variable; some cases had almost a continuous BM whereas others completely lacked a BM. Desmoplasia is a local response to the presence of tumor cells in the interstitial stroma and is characterized by excessive accumulation of stromal elements. Tumor cells might produce soluble factors that elicit a chemotactic and mitogenic response of fibroblasts or myofibroblasts (21). Alternatively, proliferation of (myo)fibroblasts may be caused primarily by the fibrin deposited in the tumor periphery (22). The predominance of the myofibroblast in tumor desmoplastic reaction has been observed in a variety of neoplasms (23-26), especially at the site of invasive growth (25, 26). These observations suggest that desmoplasia might be a reaction to invasive growth. In line with this assumption we found BM deposition in desmoplastic areas to be highly variable and discontinuous. In a recent study Barsky et al (27) demonstrated in a syngenic mouse melanoma model that treatment of the animal with

L-3,4-dehydroproline, which blocks the formation of collagen, leads to increased incidence of metastasis. Microscopically in these animals the desmoplastic reaction to the tumor appeared to be strikingly reduced. These results strongly suggest the existence of a stromal defence mechanism against tumor invasion and subsequent metastasis. Dvorak et al (22), however, reported that collagen synthesis is a relatively late event in the desmoplastic reaction. In our opinion, the temporal discordance between collagen synthesis and invasion and metastasis does by no means rule out a positive influence of early desmoplasia on these precursors.

A relationship between the grade of differentiation and the amount of BM deposition at the tumor/stromal border in the central regions of the tumors was not found. However, the number of studied cases was too low for reliable statistical analysis. For this reason we have extended the number of cases and will report about the relation between grade of differentiation and BM deposition in the future. Immunohistochemical studies of colonic adenocarcinomas revealed an almost complete lack of BM in poorly differentiated tumors and extensive BM deposition in well differentiated tumors (5, 28). In contrast to these findings, we did not find a significant correlation between the degree of differentiation and BM deposition in colorectal carcinomas in a recent study (6).

We conclude that electron microscopic and immunohistochemical studies of the role of BM in invasive tumor growth in the lung yield comparable and reliable results. The periphery of bronchogenic squamous cell carcinoma shows expansive growth only, invasive growth being restricted to the tumor center. Myofibroblasts might play a crucial role in the desmoplastic reaction in the tumor center. Finally the occurrence of seemingly intracytoplasmic BM immunoreactivity is probably caused by engulfment of BM by tumor cells.

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CHAPTER 6

Basement membrane deposition in benign and malignant nevo-melanocytic lesions: An immunohistochemical study with antibodies to basement membrane components.*

6.1 INTRODUCTION

During the complex processes involved in tumor invasion and metastasis, tumor cells have to traverse extracellular matrix barriers. Basement membranes (BM) form the boundary between the epithelial compartment and adjacent stroma. Transition from in situ to invasive carcinoma implies BM breakdown and passage of tumor cells through the BM (1). The situation is even more complex, however, as interactions between tumor cells and extracellular matrix can also result in induction of matrix synthesis by stromal cells and production of matrix components by tumor cells (2). Since the development of specific antibodies to BM components, reliable light microscopic immunohistochemical visualization of BM is possible. Immunohistochemical staining of BM has proved to be helpful to differentiate between benign and malignant lesions: discontinuities usually being limited to malignant lesions (1, 3). However, exceptions have been found to this rule: BM discontinuities do occur in benign neoplasms and conversely, intact BM have been reported in malignant tumors (3). Furthermore, inflammatory infiltrate adjacent to BM may be responsible for BM discontinuities (4).

The diagnosis of nevo-melanocytic lesions is often a problem in diagnostic histopathology. The distinction between nevocellular nevi, dysplastic nevi and malignant melanoma may be difficult (5,6). Juvenile melanoma for example is a benign lesion, which morphologically resembles malignant melanoma (5). Assessment of the depth of invasion is prognostically important (7, 8) but, especially in the presence of inflammatory infiltrate, it can be difficult. Angioinvasive growth is another prognostic indicator but its assesment is complicated by artefacts due to tissue shrinkage by fixation and embedding. The use of BM immunohistochemistry may provide (partial) solutions to these problems.

Using reticulin impregnation (Gordon and Sweet method) it was reported that completely intact BM occur around benign nevo-melanocytic lesions. However, in borderline cases reticulin staining appeared to be of little diagnostic use (9, 10). One of the reasons may be that reticulin impregnation, besides BM, also stains other structural proteins (11). Alternatively, BM deposition in nevocellular nevi and malignant melanomas has been studied at the ultrastructural level (12). Further-

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Submitted to Histopathology.

more, two immunohistochemical studies on nevo-melanocytic lesions, using antibodies to BM components have been published (13, 14). One of these studies reported a complete lack of immunoreactivity to BM components in 50% of malignant melanomas (13). The other study, however, reported BM immunostaining in all cases, althought to a variable extent (14). BM discontinuities could be observed sporadically in some benign lesions in both studies. Reviewing these studies, it seems that the pattern of BM deposition in nevo-melanocytic lesions is not an unequivocal discriminator between benign and malignant lesions.
To clarify the conflicting results in the literature, we studied a series of nevo-melanocytic lesions by immunohistochemistry, using antibodies to the BM components type IV collagen and laminin. In addition to assessing the integrity of the BM we looked for characteristic staining patterns, which could be helpful in distinguishing between benign and malignant lesions. Also the use of BM immunostaining in the detection of angioinvasive growth was studied. Furthermore the influence of the inflammatory infiltrate in BM deposition was analyzed. Finally, we compared BM deposition in the metastasis of malignant melanomas with BM staining in the primary tumors.

6.2 MATERIAL AND METHODS

6.2.1 Case material

From the files of our department 55 cases of malignant melanoma and 45 cases of benign nevo-melanocytic lesions were collected randomly (Table I and II). Metastases of malignant melanomas were also included in this study. Malignant melanomas were classified in conformity with the revision of the Sydney classification (15).

Table I

Malignant melanoma with adjacent component of:			
	type	# cases	# metastases
I:	superficial spreading type	22	1
II:	lentigo maligna type	5	1
III:	acral lentigenous type	6	—
IV:	mucosal lentiginous type	—	—
V:	no adjacent component (nodular malignant melanoma)	13	3
VI:	of unclassifiable histogenetic type	9	1
	Total #	55	6

Table II

Benign nevo-melanocytic lesions:	# cases
1. lentigo simplex	5
2. lentigo senilis	5
3. junction nevi	5
4. compound nevi	5
5. dermal nevi	5
6. halo nevi	5
7. juvenile melanoma	5
8. dysplastic nevus	5
9. blue nevus	5
Total #	45

6.2.2 Immunohistochemistry

All tissue specimens were routinely fixed in 4% formaldehyde and embedded in paraffin. Paraffin sections were deparaffinized, rehydrated, blocked for endogenous peroxidase activity with 0.3% H_2O_2 in methanol and washed 3x5 min in PBS. Subsequently tissue sections were incubated with 6M guanidine-HCl in 50 mM Na-acetate pH 6.5 as described extensively elsewhere (16), washed 3x10 min with PBS and incubated with 0.1% pepsin (Boehringer) in 1N HCl, 60 min at RT. Subsequently the sections were washed 3x5 min in PBS and incubated with polyclonal antibodies to type IV collagen 1:200 diluted serum (diluted in PBS with 0.1% BSA), or with polyclonal antibodies to laminin diluted 1:100 in (PBS with 0.1% BSA). Both antisera were raised in rabbits, immunospecificity of these antisera has been reported extensively elsewhere (17,18). Visualization was realized by an indirect immunoperoxidase technique with diaminobenzidine-H_20_2 or in heavily pigmented lesions with aminoethylcarbazole as substrate. The intensity of diaminobenzidine precipitate was enhanced by adding imidazol to the substrate.

6.3 RESULTS

6.3.1 Immunohistochemistry

The reliability of the immunostaining was judged in the adjacent normal skin, which had to show staining of BM of the epidermis, skin appendices, vessels and nerves in order to be accepted. To expose the antigenic sites for the polyclonal antisera to type IV collagen and laminin in neutral buffered formaldehyde fixed and paraffin embedded tissues, pepsin digestion was used. In the majority of the studied specimens, however, the obtained immunoreactivity was not sufficient. The im-

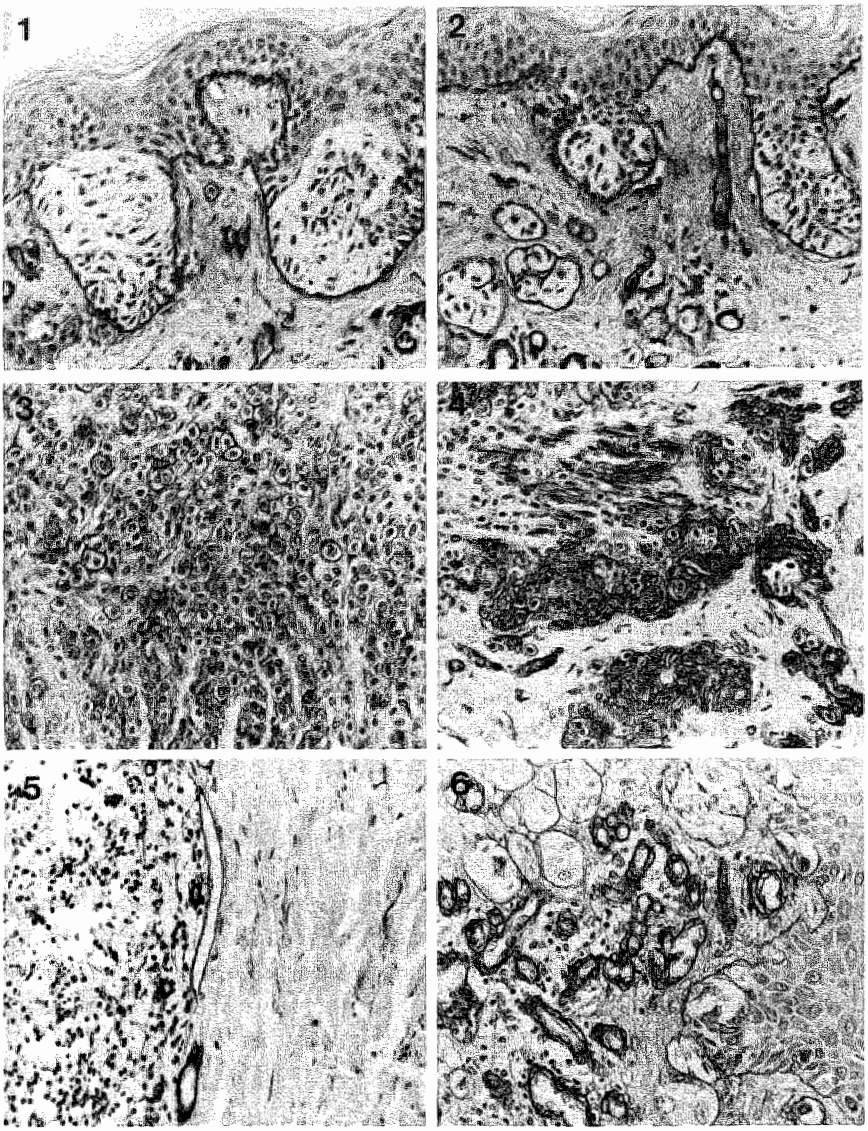


Fig. 1-15. Paraffin sections of nevo-melanocytic lesions reacted with polyclonal anti type IV collagen antibodies (immunoperoxidase).

Fig. 1. Continuous BM at the epidermal-dermal junction in a junction nevus (235x).

Fig. 2. Compound nevus: intercellular BM deposition in dermal cell nests (235x).

Fig. 3. Dermal nevus: pericellular BM deposition with a variable intensity (235x).

Fig. 4. Dermal nevus with BM deposition around spindle cells(235x).

Fig. 5. Halo nevus with rests of BM within the inflammatory infiltrate as well as the adjacent dermis (235x).

Fig. 6. Juvenile melanoma: small cell nests surrounded by a BM and intercellular BM deposition (235x).

munoreactivity could be completely restored by preincubation with 6M Guanidine-HCl in 50 mM Na-acetate buffer pH 6.5. In general, the staining intensity with the anti laminin antiserum was identical to that with the anti type IV collagen antiserum, but somewhat weaker. Laminin reactivity could not be restored in all the tissue specimens.

6.3.2 Benign nevus cell tumors

In lentigo simplex and lentigo senilis the epidermal BM showed complete continuity with BM staining for type IV collagen and laminin, comparable to that in normal skin.

Junction nevi showed large cell nests in the basal part of the epidermis, which were totally enclosed by the continuous epidermal BM (Fig 1). However, there was some variation in the intensity of the epidermal BM staining (Fig 1). Intercellular immunostaining could not be observed within the nevus cell nests.Compound nevi also showed a continuous epidermal BM with some variability of staining intensity. Nevus cell nests just below the epidermal BM were surrounded by a continuous BM (Fig 2). In contrast to the intra-epidermal cell nests, the dermal cell nests showed intercellular immunoreactivity (Fig 2). Smaller cell nests, also surrounded by a continuous BM were in the majority of cases located in the deeper parts of the dermis and showed less intercellular immunoreactivity. In dermal nevi, the larger cell nests with intercellular immunoreactivity were, as in compound nevi, mostly located just below the continuous epidermal BM. Individual nevus cells, which were also detected in compound nevi, showed a pericellular immunoreactivity, highly variable in intensity and even occasionally multilayered (Fig 3). Nevus cells with pericellular immunoreactivity were mostly located in peripheral parts of the lesions, deep in the dermis. They were surrounded by stroma, or lay close together, resembling peripheral nerve tissue, or arrector pili muscle (Fig 4). Junction, compound and dermal nevi showed, at the site of lymphocytic infiltrate, sporadically slightly discontinuous BM. Halo nevi lacked, at the site of intense lymphocytic infiltrate, any immunoreactivity to BM components at the border of nevus cells, and showed discontinuity of the epidermal BM. Rests of BM were found in the inflammatory infiltrate and also in deeper parts of the dermis between collagen fibers (Fig 5).

In juvenile melanoma, clusters of nevus cells as well as multinucleated and individual nevus cells showed almost continuous but variable immunoreactivity, at the border of nevus cells and stroma (Fig 6). The clusters showed linear or sporadically granular intercellular immunoreactivity. Hyaline bodies located at the dermal-epidermal junction showed also immunoreactivity for laminin and type IV collagen (Fig 7).

Blue nevi of the common type showed irregular pericellular immunoreactivity of the slender, slightly wavy cells. Between collagen fibers neighbouring these cells, BM deposition was found. In addition to these spindle cells, nevus cells with abundant

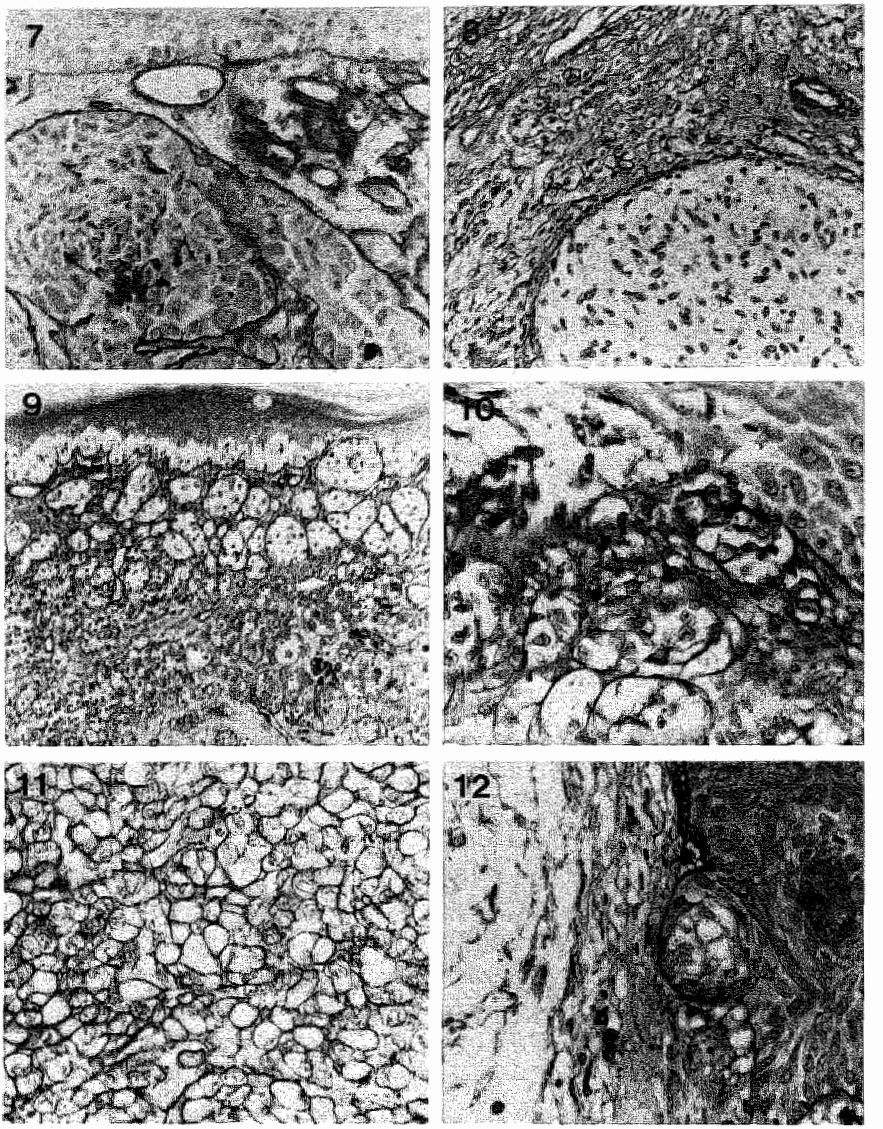


Fig. 7. Juvenile melanoma expressing immunoreactivity at the side of the "hyaline" bodies (235x).

Fig. 8. Cellular blue nevus: the epithelial part shows only at the stromal border BM deposition, individual cells in the periphery deposit pericellular BM (235x).

Fig. 9. Superficial spreading melanoma showing large clusters the epidermal-dermal junction (120x).

Fig. 10. Discontinuous BM at the epidermal-dermal junction in superficial spreading melanoma (375x).

Fig. 11. Pericellular BM deposition in superficial spreading melanoma (375x).

Fig. 12. Continuous BM at the epidermal-dermal junction in lentigo maligna (375x).

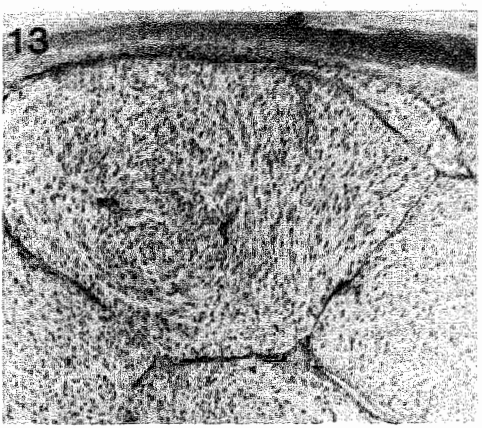
cytoplasm and continuous pericellular immunoreactivity could be identified. Cellular blue nevi lacked in the central regions immunoreactivity, however peripheral regions showed pericellular BM deposition (Fig 8).

6.3.3 Malignant melanoma with an adjacent component

I: Superficial spreading type. Large clusters of tumor cells were mostly located at the dermal-epidermal junction (Fig 9). These clusters showed intercellular immunoreactivity. In contrast, intercellular BM deposition was not found in tumor cell clusters located in the epidermis. Discontinuities in the epidermal BM in the absence of inflammatory infiltrate could be detected, but varied between limited and moderate (Fig 10). At the sites of regression, the discontinuity of the BM was extensive. Pericellular immunoreactivity was detected in large tumor cell fields (Fig 11), as well as around individual tumor cells surrounded by stroma.
II: Lentigo maligna type. Specific patterns could not be detected. As in I the large cell clusters were preferentially located at the dermal- epidermal junction but small clusters were also present. In the absence of inflammatory infiltrate the epidermal BM was continuous in adjacent lentigo maligna (Fig 12). BM deposition at the junction was as in I.
III: Acral lentiginous type. These lesions showed identical findings as in I and II. The adjacent epidermis, with atypical melanocytes in the basal cell layer, showed a continuous BM.
IV: Mucosal lentiginous type was not studied.
V: No adjacent component, also designated nodular malignant melanoma. The majority of these tumors had an architecture of large fields of tumor cells (Fig 13). Especially around small vessels in these tumor cell fields inter- and pericellular BM deposition was found. However, in some tumors patterns as described in I could be identified.
VI: Unclassifiable histogenetic type. As in V, BM deposition occured mostly around large fields of tumor cells. Some tumors showed limited BM deposition in the absence of inflammatory infiltrate, mostly in the deeper parts of the tumor.

6.3.4 Additional findings in malignant melanomas

Tumor cell clusters surrounded by BM were found in vascular lumina (Fig 14). All metastases of malignant melanoma showed BM deposition. Comparison of the pattern in the primary tumor and metastases revealed a variation from almost identical to largely different. Very extensive BM deposition could even be detected in a lymphnode metastasis of malignant melanoma (Fig 15).



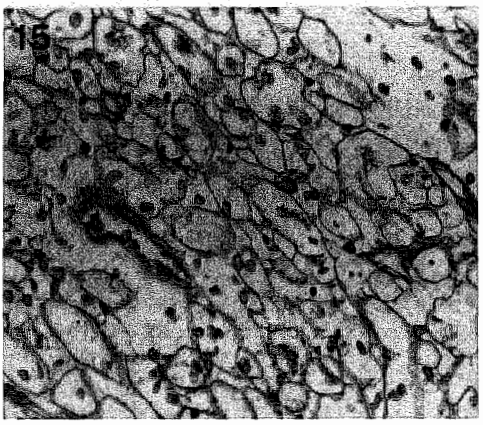


Fig. 13. Nodular malignant melanoma showing large tumor cell fields surrounded by BM (95x).
Fig. 14. Malignant melanoma cells, located in a vessel and surrounded by a BM (375x).
Fig. 15. Lymphnode metastasis of malignant melanoma: BM deposition around individual cells and small clusters (375x).

6.4 DISCUSSION

This immunohistological study on archival material of nevo-melanocytic lesions demonstrates that retrospective studies of BM deposition can be reliably performed, provided that appropriate treatment of tissue sections restores the immunoreactivity lost during tissue fixation and embedding. The guanidine-HCl procedure appeared to be an optimal approach with regard to type IV collagen immunoreactivity.
From the point of view of tumor biology the most important finding of the present study was that in almost all nevo-melanocytic lesions BM were found at the border-

line between nevo-melanocytic cell clusters (or even individual cells) and the surrounding connective tissue but not between keratinocytes and nevomelanocytes. In intradermal cell nests BM material was also seen between nevomelanocytes. Continuous BM were even seen around intraluminal angioinvasive melanoma cells and in lymphnode metastases of melanoma. It seems therefore that interaction with extra-cellular matrix or connective tissue stromal elements plays a dominant role in the deposition of a BM. Wether or not stromal components actually contribute to the formation of a BM, as we have recently shown in human colorectal carcinoma xenografts (19), remains to be established. It has been shown, however that murine B 16 melanoma cells are capable of in vitro production of type IV collagen and laminin (20), which indicates that the basement membranes surrounding nevo-melanocytic lesions may well be a product of the tumor cells.

The biological importance for melanocytes is exemplified by the role BM play in the migration of melanocytes from the dorsal site of the neural tube to the epidermal ectoderm. This migration occurs before the BM surrounding the neural tube becomes continuous and the migrating cells remain in contact with BM (21, 22). Nevocellular nevi are commonly regarded as ectopic cell nests, which have failed to reach the epidermis. In gaining access to the epidermis migrating melanocytes have to pass through the epidermal BM, which subsequently closes again. The deposition of a BM around intradermal nevus cells can be regarded as a similar phenomenon. BM surrounding invasive melanoma cells, however, represent a different phenomenon. Melanocytes have to invade trough the epidermal BM and a neo-BM has to be deposited around the invading cell clusters. Around these, the BM frequently appeared to be interrupted, which was never seen in nevocellular nevi except when accompanied by inflammatory infiltrate.

It has been shown that murine melanoma cell lines with high metastatic capability have a higher tendency to produce BM degrading type IV collagen specific collagenase than melanoma cells with low metastatic capability (23). This observation would suggest that melanoma with extensive BM interruptions would show a higher incidence of metastasis than melanoma with (almost) continuous BM. Although to date we have not been able to extensively analyze this possibility we have not found any evidence to support such a correlation.

Earlier studies of BM in melanomas have yielded conflicting results. Natali et al. (13) found absence of immunoreactivity for BM components in 50% of their cases. In contrast Stenbäck et al. (14) found BM deposition in all melanomas, although to a variable extent and discontinuous. Our findings are in agreement with the latter. Almost all melanomas contained extensive BM deposition throughout, although frequently interruptions occured. Differences between tumor center and periphery, as we have reported for colonic adenocarcinomas and squamous cell carcinomas of the lung (24, 18), did not occur.

In connection with the histopathological diagnosis of nevo-melanocytic lesions our findings allow the following conclusions.

1. In doubtful cases interruptions of BM, in the absence of inflammatory infiltrate, is evidence in favor of invasive melanoma.
2. The pattern of BM deposition does not differ for the various categories of nevomelanocytic lesions and therefore is of no help in their histological classification.
3. The pattern of BM deposition does not differ for melanomas with varying levels of invasive growth and therefore offer no help in the assesment of the depth of invasion.
4. The hyaline bodies in juvenile melanoma (25) appear to consist of BM components (26), which is also illustrated by this study. BM immunohistochemistry facilitates their recognition and therefore may be of some help to distinguish between juvenile and true malignant melanoma.
5. Staining of the endothelial BM allows reliable identification of vascular structures in melanomas and facilitates the recognition of angioinvasive growth, which is regarded as an important prognostic variable.
6. Extensive deposition of BM around individual cell and cell nests in a poorly differentiated tumor of unknown origin is evidence in favor of melanoma but can never be used as conclusive evidence.

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CHAPTER 7

General discussion

7.1 Introduction

In this final chapter an attempt will be made to discuss the results presented in this thesis in a somewhat broader context than was aimed at in the discussion in the individual chapters. Two main themes will be focussed upon. Firstly, our findings in chapter 2 and 3 are discussed in connection with the general aspects of BM structure, composition and metabolism, especially in regard of tumor invasion. Secondly, the impact of BM immunohistochemistry on diagnostic histopathology is reviewed.

7.2 BM STRUCTURE AND COMPOSITION

The work presented in chapters 2 and 3, illustrates that immunohistochemical identification of basement membranes (BM) forms a powerful tool in the study of cell-extracellular matrix interactions. However, detection of immunoreactivity with antibodies to BM components is not conclusive evidence of the presence of a structured BM, which would require electron microscopic confirmation. Electron microscopy allows detailed structural analysis of subcellular structures, cellular interactions and extracellular matrix. The limited sample size for electron microscopy is, however, a significant disadvantage. In contrast, immunohistochemistry provides immunochemical evidence of the chemical nature of tissue structures, on relatively large samples but with little structural resolution. The combination of both techniques, immuno-electron microscopy, would significantly improve our understanding of structure and composition of BM in normal and neoplastic tissues (1-4). Another limitation of immunohistochemistry is that a positive finding only indicates the presence of the substance of interest but provides no information concerning the dynamics of production and degradation. For BM such additional information might be obtained through metabolic labelling studies, combining immunohistochemistry with autoradiographic detection of incorporated labelled precursors (5). An alternative approach would be detection of BM component specific mRNA's by hybridization histochemistry (6).
In addition to localization, application of BM component specific antibodies has facilitated studies concerning the interactions between BM component specific sites on cell surfaces and specific domains on the macromolecules which constitute the BM. By blocking well defined regions on BM molecules with monoclonal antibodies functional domains have been discovered (7). The characterization of plasma mem-

brane associated proteins such as the laminin receptor (8-10), which specifically interact with BM and interstitial proteins has started recently. Biochemical and molecular biological studies will reveal new, possibly tissue and organ specific, constituents of BM and interstitial stroma (11-13). Subsequently monoclonal antibodies to these components will provide a tool for functional analysis of molecular domains and immunohistochemical studies using these antibodies will give more insight into their biological significance.

7.3 BM DEPOSITION IN NEOPLASIA

The origin of BM components has not been completely elucidated. The generally held view is that the adjacent epithelial or endothelial cells synthesize the BM constituents. However, there is substantial evidence from studies, using epithelial tumor xenografts and species specific antibodies reactive with BM components, that these components may not only be derived from the adjacent epithelium but are also deposited by stromal elements (14). Immunohistochemistry may not be the optimal approach to resolve this issue. In situ hybridization with nucleic acid probes for BM component-mRNA may be a more conclusive approach to determine exactly which cells are involved in the synthesis and deposition of epithelial BM. Coculturing experiments of fibroblasts with cancer cells might be an even more dynamic approach to solve this problem.
In general, the unraveling of the mechanisms involved in BM metabolism will be strongly facilitated by in vitro models in which BM deposition of normal and tumor cells can be studied in the presence of various types of mesenchymal elements and in various artificial collagen matrices (15). Our results with tumor cells, cultured in artificial collagen lattices has so far been discouraging, because tumor cells which after xenografting into nude mice demonstrated BM deposition could not be induced to deposit BM in vitro under a variety of conditions. Production of various BM components in vitro has often been detected, but only a few studies concerning assembly of BM components to a BM-like structure at the border of epithelial cells and artificial stroma have been reported (16). Additives to standard tissue culture medium, such as vitamins, growth factors, hormones, and inhibitors for BM degrading enzymes might be an approach to obtain BM deposition in vitro.

7.4 BM AND TUMOR INVASION

Studies on BM structure and turnover, might be important in cancer research. In vitro and in vivo manipulation of cell-extracellular matrix interactions with monoclonal antibodies to BM constituents might yield important information concerning the mechanisms involved in tumor invasion. BM in tumors are probably even more than in normal tissues dynamic structures, the immunohistochemical

staining showing the positive balance between BM deposition and BM degradation. Degradation of BM requires specific enzymes such as type IV collagenase (17), along with many other proteases involved in this process (18). How exactly tumor cells in invasive neoplasms degrade and pass through BM has not been fully elucidated. Similarly, how intravascular potentially metastasizing tumor cells lodge at the metastatic site and cross the local BM, before they reach the interstitial compartment in distant organs has not been elucidated. In vitro and vivo studies of the passage of leukocytes through BM might be a readily available model and as such be helpful in unraveling the role of BM degrading enzymes during the passage of cells through a BM (19).
Activators and inhibitors of degrading enzymes presumably are involved in the regulation of BM degradation (20). BM degrading enzymes have been isolated from tumors and their metastases as well as from tumor cell-lines. High levels of these enzymes correlated with increased invasive and metastatic capability (21, 22). Specific antibodies to BM degrading enzymes might serve to identify specifically which cells are involved in the invasive process (23), assuming that not all cells in a malignant neoplasm have invasive capacities. Such studies, however, will not reveal the influence of interstitial stromal elements on the turnover of BM. On the whole the role of stromal elements in the invasive process appears to be underrated.

7.5 BM IMMUNOSTAINING IN DIAGNOSTIC HISTOPATHOLOGY

In the second part of this general discussion we will review the significance of BM immunohistochemistry for diagnostic histopathology of neoplasia. Conventionally, BM visualization has been achieved through application of staining techniques such as reticulin silver impregnation or PAS staining. These techniques, however, are not specific for BM structures. Reticulin stains impregnate reticulin fibers as well as BM whereas PAS staining demonstrates all glycoproteins, including most BM components (24). In recent years the conventional histochemical stains have been replaced by BM immunostaining, using monospecific antibodies against type IV collagen and laminin. An important potential application would be the distinction between invasive and non invasive neoplastic lesions, which is a major problem in diagnostic histopathology. Based upon a large number of observations it can be concluded that in general normal epithelia as well as dysplastic and borderline non-invasive lesions display a continuous BM, whereas in invasive carcinomas BM are discontinuous (23, 25). This difference can not be used in lesions with an intense inflammatory infiltrate, which is also accompanied by discontinuities of the BM (26). Assessment of invasive growth by BM immunostaining appears to be more helpful in neoplastic lesions originating in surface epithelia than in the epithelium of parenchymatous organs. Especially in non invasive lesions of the breast, urinary bladder, uterine cervix, and squamous epithelia BM immunostaining is of diagnostic importance (25, 27). However, BM discontinuity may never be used as a single decisive criterium of

invasive growth. Highly differentiated squamous cell carcinomas usually deposit continuous BM and, conversely, inflammatory infiltrate can produce discontinuities in non invasive lesions through degradation of the BM presumably by proteases released by leukocytes. In the presence of inflammatory infiltrate, therefore, it may be impossible to distinguish between carcinoma in situ and invasive carcinoma. Another example of a frankly invasive neoplasm which nevertheless can be demarcated by a continuous BM is malignant melanoma (28).

BM immunostaining can also be useful for the determination of the nature of poorly differentiated tumors. In the absence of BM deposition at the border of tumor cells and surrounding connective tissue, a diagnosis of carcinoma or of sarcoma, originating from cells which are normally surrounded by a BM such as Schwann cells, muscle cells and fat cells, is rather unlikely. There are, however, exceptions to this general rule. Poorly differentiated leiomyosarcoma and monophasic (fibrous) synovial sarcoma may lack any immunoreactivity to BM constituents (29). Nevertheless pericellular immunoreactivity appears to be a characteristic of soft tissue tumors originating from cells which are normally surrounded by BM. More specifically, fibrosarcomas and malignant fibrous histiocytomas are characterized by the complete absence of BM immunoreactivity (29).

A non-specific but nevertheless rather useful feature of BM immunostaining is its elucidation of the architectural characteristics of a tumor. The architecture in papillary, tubular or alveolar patterns can be readily identified, even in necrotic areas and the pattern of tumor vascularization can also be easily visualized. This latter feature facilitates the recognition of angio invasive growth. Although lymph capillaries show a discontinuous BM or are totally devoid of BM larger lymph vessels deposit a continuous subendothelial BM. Because lymph capillaries lack immunoreactivity with antibodies to factor VIII, BM immunostaining may assist in the recognition of lymph vessels. Blood capillaries, however, are clearly demarcated by BM immunohistochemistry which make them more readily recognizable than by immunostaining with antibodies to factor VIII (30).

Some neoplasms can be recognized by a characteristic pattern of BM immunostaining. A typical example is the occurrence of extracellular hyaline bodies in adenoid cystic carcinoma, endodermal sinus tumor, juvenile melanoma and odontogenic cysts which express immunoreactivity with antibodies to type IV collagen and laminin (31). Furthermore, in adenoid cystic carcinoma extremely thick BM deposits occur around tumor cell nests (32).

7.6 CONCLUDING REMARKS

The future will tell what role antibodies to BM specific antigens will ultimately play in the routine histopathological diagnosis of neoplastic lesions. It is beyond doubt, however, that the study of the structure, composition (including identification of new cell- and tissue specific BM components) and metabolism of the BM and its in-

teractions with adjacent epithelial and stromal cells are of tremendous importance in the elucidation of the significance of the extracellular matrix for normal and neoplastic cellular behaviour.

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SUMMARY

In this thesis some aspects of the interaction between tumor cells and the extra cellular matrix, as reflected in the basement membrane (BM) are studied.
Chapter 1 introduces the problem. The structure and composition of the BM is reviewed. It is emphasized that, although the biochemistry and molecular biology of the major components common to all BM, type IV collagen and laminin, have been clarified, many questions have remained unanswered. The dynamics of BM, for example, and the nature and function of cell or organ specific BM components are largely unknown.
In this chapter furthermore the role of BM in neoplasia is reviewed. Originally BM were regarded as static structures which were destroyed by the invading malignant tumor cells. It is now clear that tumor cells not only degrade BM components, but also deposit BM. Consequently, the patterns of BM deposition in neoplasms are the result of a dynamic balance between deposition and BM degradation. Which factors regulate this balance in neoplastic cells to date remains largely unknown.
In diagnostic histopathology immunohistochemical staining for BM components has been used to distinguish invasive from non invasive neoplasms, for the classification of soft tissue tumors, and as a prognostic indicator. In the final part of this chapter the aim of the studies described in this thesis is outlined.
Chapter 2 describes two new monoclonal antibodies reactive with human type IV collagen in frozen as well as routinely fixed and processed tissue sections. The antibodies (1042 and 1043) were raised against human placental type IV collagen and were shown by immunoblotting and ELISA tests to react exclusively with type IV collagen determinants. Extensive immunohistochemical survey studies on panels of tissues from various species, using unfixed cryostat sections, demonstrated that antibody 1043 reacted only with human type IV collagen whereas antibody 1042 in addition reacted with rabbit type IV collagen. All tissues showed homogeneous staining of the basement membrane, indicating that the detected epitopes did not show organ-specific distribution.Tissue processing protocols for using these monoclonal antibodies on routinely processed paraffin embedded tissues were developed. It was found that whereas polyclonal anti-type IV collagen antisera required pepsin digestion, our monoclonal antibodies required pronase or papain digestion to restore type IV collagen immunoreactivity in paraffin sections. It is concluded that these monoclonal anti-type IV collagen antibodies detect species specific epitopes which can be detected in routinely processed paraffin embedded tissues after appropriate enzyme pretreatment.
In chapter 3 the origin of BM at the tumor-stromal interface was studied in nude

mouse and rat xenografts of human tumor cell-lines. Polyclonal cross-species reactive anti type IV collagen antibodies and two monoclonal human specific type IV collagen antibodies, as described in chapter 2, were used. The xenografts were derived from a colonic adenocarcinoma (5583-S), transformed human amnionic epithelium (WISH), and a human oral epidermoid carcinoma (KB). With the cross-species reactive anti type IV collagen antibodies, BM were identified, in the original colonic adenocarcinoma and in all xenografts, at the tumor cell stromal border as well in the tumor vasculature BM. The human specific antibodies visualized the same pattern of type IV collagen deposition in the original colonic adenocarcinoma and in xenografts of normal human colonic mucosa, whereas in xenografts of 5583-S neither at the tumor/stromal border nor in the vasculature BM immunostaining was obtained. In vitro 5583-S cells did not produce any type IV collagen. Xenografts of WISH and KB cells showed discontinuous BM immunoreactivity at the tumor-stromal border with cross-species reactive as well as human specific antibodies. Vascular BM, however, failed to show immunoreactivity with human specific antibodies to type IV collagen. In vitro in these cells production of type IV collagen, including the specific human epitope, could be demonstrated. These observations strongly suggest that BM at the tumor/stroma interface in xenografts of 5583-S cells are of murine origin. In contrast, the BM in xenografts of WISH and KB cells is at least partly of human origin. Therefore, mesenchymal stromal elements appeared to be involved in the production and extracellular deposition of BM components in epithelial neoplasms.

In chapter 4 BM deposition at the tumor-stromal border was studied in 163 cases of colorectal carcinomas. Type IV collagen immunoreactivity was scored semiquantitatively as moderate/extensive versus limited and these scores were correlated with Dukes' stages and survival data. Cases with limited BM deposition showed a significantly shorter overall survival. Stratification of the cases for limited versus moderate/extensive BM deposition and Dukes' stages A/B versus C/D, showed that in Dukes' stages C/D, cases with moderate/extensive BM deposition showed a significantly better survival than cases with limited BM deposition. Our results suggest that immunostaining of BM of cases in Dukes C differentiates tumors with relatively high invasive and metastatic capacity from tumors with low invasive and metastatic capacity.

In chapter 5 BM deposition at the interface of tumor cells and stroma was studied in 27 bronchogenic squamous cell carcinomas. From peripheral and central parts of the tumors, specimens were collected and frozen as well as formalin fixed and paraffin embedded. Also specimens for electron microscopy were collected. Using antibodies to type IV collagen and laminin, the BM was visualized in light microscopy, by an indirect immunoperoxidase technique. Light microscopic findings were compared to ultrastructural observations. The peripheral parts of the tumors showed continuous BM in a recognizable preexisting alveolar pattern, without evidence of invasive growth into the alveolar septa. In contrast, central parts showed

highly variable BM deposition, ranging from continuous to almost completely absent. Alveolar patterns were not observed in the tumor center. The stromal compartment of the tumor center contained many spindle cells with irregular pericellular BM, which could ultrastructurally be identified as myofibroblasts. Electron microscopy and immunohistochemistry yielded comparable results. It is concluded that the periphery of bronchogenic squamous cell carcinomas show expansive growth. Invasive growth appears to be restricted to the tumor center.

Patterns of BM deposition were furthermore investigated in benign and malignant nevo-melanocytic lesions by BM immunostaining as described in chapter 6. To restore the immunoreactivity with antibodies to BM components, paraffin sections from stored tissue blocks required pretreatment with 6 M guanidine-HCl in addition to pepsin preincubation. BM deposition was found around clusters as well as individual nevo-melanocytic cells in contact with dermal stroma. However, between keratinocytes and completely intra-epidermally located nevo-melanocytic cells, BM immunostaining could not be detected. Tumor cell-stromal interaction apparently is a prerequisite for BM deposition in nevo-melanocytic lesions. BM discontinuities in the absence of inflammatory infiltrate appeared to be evidence in favor of malignant melanoma in doubtful cases. The general pattern of BM deposition in benign and malignant lesions was found to be identical and therefore of no help in differential diagnosis. Identification of hyaline bodies, which show immunoreactivity with antibodies to BM components, may be helpful to distinguish between juvenile and malignant melanomas. Detection of angioinvasion, a prognostic indicator in malignant melanoma, is facilitated by BM immunostaining. Pericellular BM deposition in cell clusters in metastasis of a poorly differentiated tumor of unknown origin argues in favor of the possibility of malignant melanoma.

In chapter 7, the results of the studies are discussed against the background of general aspects of BM in relation to tumor invasion. Finally, the significance of BM immunostaining for diagnostic histopathology is reviewed.

SAMENVATTING

In dit proefschrift zijn enkele gezichtspunten van de wisselwerking tussen tumor cellen en extracellulaire matrix, zoals weerspiegeld in basale membranen (BM), het onderwerp van studie.
In hoofdstuk 1 wordt een overzicht van de structuur en samenstelling van de BM gegeven. Ondanks de opheldering van vele biochemische en moleculair biologische aspecten van belangrijke BM componenten zoals collageen type IV en laminine, blijven nog vele vragen onbeantwoord. Over de aanmaak en afbraak van BM is, evenals over de aard en functie van cel- en orgaan specifieke bestanddelen van de BM, weinig bekend.
Verder wordt in dit hoofdstuk een overzicht gegeven van de rol die BM spelen in neoplastische lesies. Aanvankelijk werden BM beschouwd als statische structuren die worden doorbroken door invasief groeiende maligne tumor cellen. Momenteel is duidelijk geworden dat tumor cellen niet alleen BM componenten afbreken, maar ook in staat zijn tot het afzetten van BM. Hieruit kan worden afgeleid dat de patronen waarin BM in neoplastische aandoeningen voorkomen, de resultante zijn van aanmaak en afbraak. Over de manier waarop deze evenwichts-situatie in tumoren in stand wordt gehouden is op dit moment nog weinig bekend.
In de diagnostische histopathologie wordt de immunohistochemische aankleuring van BM toegepast om invasieve van niet invasieve tumoren te onderscheiden. Verder is deze techniek van belang voor de differentiële diagnostiek van weke delen tumoren en heeft de mate van BM depositie prognostische betekenis. In het laatste gedeelte van hoofdstuk 1 wordt de vraagstelling van dit proefschrift geschetst.
Hoofdstuk 2 beschrijft twee monoclonale antilichamen gericht tegen humaan collageen type IV, die zowel toepasbaar zijn op ingevroren als op in paraffine ingebed weefsel. De antilichamen (1042 en 1043) werden opgewekt tegen uit humane placenta geïsoleerd collageen type IV, de specificiteit werd aangetoond door middel van immunoblotting en ELISA. Door gebruik te maken van een weefselpanel samengesteld uit weefsels van verschillende zoogdier species kon op coupes van ingevroren niet gefixeerde weefselstukjes worden aangetoond dat de antilichamen alleen met humane weefsels reageren. Alle humane weefsels vertoonden aankleuring van BM, waardoor orgaan specificiteit van deze antilichamen uitgesloten kon worden. Voor het verkrijgen van immunoreactiviteit in formaline gefixeerde en in paraffine ingebedde weefsels was een preincubatie met enzymen noodzakelijk. Voor het polyclonale antiserum was een pepsine digestie vereist, voor de monoclonale antilichamen pronase. Met deze monoclonale antilichamen kunnen in routinematig gefixeerde en ingebedde weefsels na enzym behandeling specifieke humane epitopen worden aangetoond.

In hoofdstuk 3 wordt de origine van BM op het grensvlak van tumor epitheel en stroma onderzocht in xenotransplantaten van humane tumor cel lijnen in immuungecompromiteerde muizen en ratten. Hiervoor werd gebruik gemaakt van polyclonale kruisreagerende anti collageen type IV antilichamen en de twee humaan specifieke anti collageen type IV antilichamen, die beschreven werden in hoofdstuk 2. De xenotransplantaten waren afkomstig van een adenocarcinoma van het colon (5583-S), getransformeerd humaan amnion epitheel (WISH), en een humaan plaveiselcel carcinoma (KB). Met de kruisreagerende polyclonale antilichamen tegen collageen type IV werden BM aangetoond op het grensvlak van tumor epitheel en stroma en in de vaatstructuren, zowel in het primaire adenocarcinoma van het colon als in alle xenotransplantaten. De humaan specifieke antilichamen lieten het zelfde patroon zien van BM depositie in het primaire adenocarcioom van het colon en in de xenotransplantaten van normale colon mucosa. Xenotransplantaten van 5583-S daarentegen vertoonden geen BM op het grensvlak van tumor en stroma, ook vasculaire BM konden niet worden aangetoond. In vitro vertoonden 5583-S cellen evenmin aanwijzingen voor productie collageen type IV. Xenotransplantaten van WISH en KB cellen vertoonden echter discontinue BM immunoreactiviteit op het grensvlak van tumor epitheel en stroma met kruisreagerende en humaan specifieke antilichamen. Vasculaire BM daarentegen lieten geen immunoreactiviteit zien met humaan specifieke antilichamen tegen collageen type IV. In vitro kon in deze cellen productie van collageen type IV worden aangetoond, zelfs de humaan specifieke epitopen werden aangetoond. Uit deze waarnemingen kan worden geconcludeerd dat de BM op het grensvlak van tumor en stroma in de xenoplantaten van 5583-S van muis of rat origine zijn. BM in xenotransplantaten van WISH en KB cellen, daarentegen zijn zeker gedeeltelijk van humane origine. Stroma cellen lijken op grond van deze bevindingen betrokken te zijn bij de productie en extracellulaire depositie van BM membraan componenten in epitheliale neoplasmata.
In hoofdstuk 4 wordt de depositie van BM op het grensvlak van tumor en stroma in 163 colorectale carcinomen besproken. De immunoreactiviteit werd semiquantitatief gescoord en ingedeeld in matig/sterk versus gering. Deze score werd gecorreleerd met de Dukes' stadia en overlevings data. De patienten groep met geringe BM depositie vertoonde een significant kortere overleving. Stratificatie van de patienten met geringe versus matige/sterke BM depositie en Dukes' stadia A/B versus C/D, liet een significant kortere overleving zien van de groep patienten in Dukes' stadia C/D met geringe BM depositie in vergelijking met de patienten in Dukes' stadia C/D met matig/sterke BM depositie. Deze resultaten suggereren dat in de groep patienten in Dukes' stadium C, tumoren met een relatief sterk invasief en metastaserend vermogen kunnen worden onderscheiden van tumoren met een gering invasief en metastaserend vermogen.
In hoofdstuk 5 wordt de BM depositie op het grensvlak van tumor en stroma in 27 plaveiselcel carcinomen van de long bestudeerd. Weefselstukjes uit het centrum en de periferie van deze tumoren werden ingevroren, in formaline gefixeerd en in paraf-

fine ingebed en er werden stukjes voor electronen microscopie bewerkt. Met antilichamen tegen collageen type IV en laminine werden de BM op licht microscopisch niveau gevisualiseerd met behulp van een indirecte immunoperoxidase techniek. De licht microscopische bevindingen werden vergeleken met de waarnemingen op ultrastructureel niveau. De periferie van de tumoren vertoonde continue BM, in het patroon van de preexistente alveolaire architectuur. Invasieve groei in de alveolaire septa werd in de periferie van de tumoren niet waargenomen. In tegenstelling hiermee, vertoonde het centrum van de tumoren een strek wisselende BM depositie, variërend van continu tot bijna totaal ontbrekend; een alveolair patroon was niet herkenbaar. Het stroma in het centrum van de tumor bevatte spoelvormige cellen met onregelmatige pericellulaire BM, die ultrastructureel als myofibroblast konden worden geïdentificeerd. De licht en electronen microscopische bevindingen stemden grotendeels met elkaar overeen. Uit deze studie kan worden geconcludeerd dat in de periferie van plaveiselcel carcinomen in de long expansieve groei plaats vindt, en invasieve groei beperkt lijkt te blijven tot het centrum van de tumor.
Het patroon van BM depositie werd ook onderzocht in benigne en maligne nevomelanocytaire lesies zoals beschreven in hoofdstuk 6. Om immunoreactiviteit te verkrijgen met antilichamen tegen BM componenten in paraffine coupes van archief materiaal was het noodzakelijk om behalve de gebruikelijke pepsine preincubatie de coupes ook met 6 M guanidine-HCl te behandelen. BM depositie werd waargenomen rond individuele- en clusters nevo-melanocytaire cellen, die in contact zijn met het dermale bindweefsel. Tussen epidermale cellen en geheel intra-epidermaal gelegen nevo-melanocytaire cellen werd geen immunoreactiviteit tegen BM componenten waargenomen. Interactie van tumor cellen met stromale componenten is klaarblijkelijk vereist voor BM depositie in nevo-melanocytaire lesies. BM discontinuïteit in de afwezigheid van ontstekings infiltraat wijst in geval van twijfel sterk in de richting van een maligne melanoma. Het algemene patroon van BM depositie is in benigne en maligne nevo-melanocytaire lesies gelijk en heeft derhalve geen betekenis in de differentiële diagnostiek. Het aantonen van hyaline deposities, die immunoreactiviteit vertonen met antilichamen tegen BM componenten, kan het onderscheid tussen juveniele en maligne melanomen ondersteunen. Het opsporen van angioinvasieve groei, een prognostische factor van betekenis, wordt aanzienlijk verbeterd door immunohistochemische aankleuring van BM. Pericellulaire BM depositie in metastasen van slecht gedifferentieerde tumoren, dient de aandacht te vestigen op de mogelijkheid van een maligne melanoma.
In hoofdstuk 7 worden de resultaten van de studies besproken tegen de achtergrond van algemene aspecten van BM in relatie tot invasieve groei van tumoren. Tot slot wordt een overzicht gegeven van het belang van de immunohistochemische detectie van BM voor de diagnostische histopathologie.

DANKWOORD

Het onderzoek voor dit proefschrift werd uitgevoerd binnen het kader van het KWF project "Basement membranes in cancer" (project nr. RL 85-2), en werd grotendeels binnen de vakgroep pathologie verricht. Uit dit dankwoord zal blijken, dat wezenlijke bijdragen zijn geleverd door andere vakgroepen en ook door instituten buiten de Rijksuniversiteit Limburg.
Op de eerste plaats ben ik mijn promotor prof.dr. F.T. Bosman zeer erkentelijk. Beste Fré, met veel genoegen kijk ik terug op de periode, waarin ik binnen het kader van het door jouw geïnitieerde KWF project, het onderzoek voor dit proefschrift heb uitgevoerd. Niet alleen jouw kennis van zaken, maar ook jouw enthousiasme, was voor mij een grote stimulans en heeft veel respect afgedwongen. In de eindfase van mijn opleiding tot patholoog-anatoom heb ik bovendien met jouw opleiders kwaliteiten kennis mogen maken.
Ir. Jacques Cleutjens, Cor Beek en Monique Vallinga-Ummelen, met jullie heb ik het grootste deel van het onderzoek uitgevoerd. De werkzaamheden en de hiermee samenhangende discussies zijn altijd in een vriendschappelijke sfeer verlopen. Jullie bijdragen aan dit proefschrift zijn voor mij van doorslaggevende betekenis geweest.
Ook prof.dr. Jan Willem Arends heeft een grote bijdrage geleverd, niet alleen inhoudelijk, maar ook door zijn voortdurende stimulerende houding.
Margriet Pijls en Birgit Kuypers-Engelen hebben nauwgezet een groot gedeelte van de immunohistochemische kleuringen verricht.
In de beginfase zijn dr. Ton de Goeij en Edith van der Linden sterk betrokken geweest bij dit onderzoek, in een latere fase heeft dr.ir. Kees Verstijnen met ons samengewerkt. Drs. Rop Simon, destijds als student-assistent aan de vakgroep pathologie verbonden, en drs. Helma van Zandvoort ben ik zeer erkentelijk voor hun inzet.
Claire Bollen-Prevot en Marianne Rikers-Laarmans verzorgden de manuscripten, die naar de vaktijdschriften werden gestuurd.
Buiten onze vakgroep wil ik dr. Theo Wiggers, thans als chirurg verbonden aan de Daniël den Hoed Kliniek te Rotterdam, bedanken voor zijn bijdrage. Voor de statistische analyse zorgde dr. Lex Volovics. Marcia Steegmans-van Puyenbroek verwerkte de patienten gegevens in het onderzoek van de colorectale carcinomen.
Bij de fotografie heb ik veel steun gehad van de audiovisuele dienst van het Academisch Ziekenhuis Maastricht.
De samenwerking met dr. Koert Dingemans, verbonden aan het Academisch Medisch Centrum, Universiteit van Amsterdam, was voor een deel van dit proefschrift van groot belang, voor zijn bijdrage ben ik hem zeer erkentelijk. Ook dr. Sjoerd

Wagenaar, patholoog-anatoom, St. Antonius Ziekenhuis Nieuwegein, dank ik voor zijn bijdrage. Verder wil ik Ella Vaarkamp en Felie Schmitz du Moulin, verbonden aan het St. Antonius Ziekenhuis Nieuwegein en Marius van den Bergh Weerman en Peter Teeling, verbonden aan het Academisch Medisch Centrum, voor hun bijdrage bedanken.
De kritische kanttekeningen en suggesties van de leden van de promotiecommissie bestaande uit: prof.dr. G. Kootstra, prof.dr. G. Blijham, prof.dr. J. Geraedts, prof.dr. M. Mareel en prof.dr. A. Martinez-Hernandez, hebben geleid tot inhoudelijke verbeteringen.
Dr. Frans Stassen wekte mijn interesse voor wetenschappelijk onderzoek tijdens een wetenschaps stage, die deel uitmaakte van de medische studie. Een vervolg hierop vormde mijn student-assistentschap bij dr. Frans Stassen, een periode waarin ik, mede door de samenwerking met Cor Beek, met vele basale onderzoekstechnieken kennis heb gemaakt. Door mijn contacten met prof.dr. Jan Willem Arends werd gedurende mijn student-assistentschap de interesse voor de klinische pathologie gewekt. In de eerste drie jaar van mijn opleiding tot patholoog-anatoom, een periode waarop ik met veel genoegen terugkijk, heeft prof.dr. G.J.V. Swaen een solide basis gelegd. In die tijd volgde hij enthousiast het begin van mijn onderzoek bij prof.dr. Fré Bosman.
Tot slot wil ik allen, die niet met naam vermeld worden, maar wel een bijdrage aan dit proefschrift geleverd hebben, van harte bedanken.

De uitgave van dit proefschrift werd financieel ondersteund door de Stichting Bevordering Klinische Pathologie Limburg en Euro-Diagnostic b.v. Apeldoorn.

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1981-1985	Assistent geneeskundige in opleiding tot patholoog-anatoom, Academisch Ziekenhuis Maastricht, opleiders prof.dr G.J.V. Swaen, prof.dr. F.T Bosman
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